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ON THE DECOMPOSITION PRODUCTS OF EPINEPHRIN HYDRATE.¹

By JOHN J. ABEL AND R. DEM. TAVEAU.

(From the Pharmacological Laboratory of the Johns Hopkins University.)

(Received for publication, August 20, 1905.)

The empirical formula, $C_{10}H_{13}NO_3 \cdot \frac{1}{2}H_2O$, which was adopted by Abel for that member of the epinephrin series which he has called epinephrin hydrate, and which was first isolated as a free base by Takamine, has not met the approval of other investigators in this field. In view of the suggestion of Abderhalden and Bergell² that this substance should be prepared in such a manner as to preclude the possibility of oxidation, the authors have undertaken the laborious task of its preparation and purification in an atmosphere of pure hydrogen. The details of this method cannot be described here. It suffices to say that all but two of the stages in the entire process were carried out in hydrogen, the two being the mincing of the fresh glands and the filtration under pressure of the acidulated alcoholic extract of the minced glands. The hydrogen used was purified by passing it through acid and alkaline permanganate solutions. All fluids employed were boiled till free from air and allowed to cool in an atmosphere of hydrogen.

We have now prepared the substance four times in this way from perfectly fresh glands, have purified it in various ways, and have made a large number of analyses. A summary of our results is as follows:

There is a lack of agreement in the nitrogen content of specimens prepared at different times and from different lots of fresh glands.

¹ This investigation has been carried on with the aid of a grant from the Carnegie Institution.

² *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 2022, 1904.

The subjoined table gives the nitrogen content of four specimens, all of which were ash-free, and all of which had been crystallized four times. The trichloroacetic acid method¹ of preparation was employed in each case, ten grams of the acid being used to each kilogram of the minced glands.

I	II	III	IV
August, 1904. Ammonia only used as a precipitant. N—8.08 per cent. ²	October, 1904. Ammonia only used as a precipitant. Fraction: I. N—7.80 per cent. II. N—8.04 per cent. III. N—8.45 per cent.	January, 1905. Potassium carbonate used in all precipitations except the last, in which ammonia was used. N—7.34 per cent.	January, 1905. Potassium carbonate only used as a precipitant. N—7.30 per cent. N—7.44 per cent.

A study of this table shows that specimens of our substance which are ash-free and which in the absence of analytical proofs would have been thought to be equally pure, vary greatly in composition. The cause of this variability has not yet been discovered.

Specimens I and II, when subjected to repeated solution in acids and repeated precipitation therefrom with alkalis, did not retain a constant composition. *Very great and rapidly occurring changes in the nitrogen content of our substance have been observed by us under these conditions.*

Thus, in attempting to purify Specimen I (N—8.08 per cent.), we found that its nitrogen content rose to 9.49 per cent. (duplicate analysis, N—9.53 per cent.). Picric acid and ammonium picrate were used in one stage of the process of purification, and the objection may be offered that this great increase in nitrogen is due to the retention of ammonium picrate. But other specimens also showed a great increase in nitrogen content which could not be accounted for in this way. For example, Fraction I, specimen of October, 1904 (N—7.80 per cent.), was dissolved in a one-per-cent. solution of acetic acid, and in the hope of

¹ Abel, *Ber. d. deutsch. chem. Gesellschaft.*, xxxvi, p. 1841, 1903.

² All of the analytical data, from which the results given in this paper have been calculated, will be published later in a monograph to be submitted to the Carnegie Institution.

facilitating the removal of impurities the solution was heated to 60° C. and precipitated at this temperature with dilute ammonia. The precipitate was well washed by decantation with water of 60°–75° C., then transferred under exclusion of air to a filter in a suitably constructed apparatus, again washed with water of 40° C., then with alcohol and ether, and dried in a current of dry hydrogen. On analysis it was found that this treatment with very dilute acid at 60° C. caused the nitrogen of our compound to rise from 7.80 to 8.73 per cent. Again, one of the fractions of a specimen that had been repeatedly crystallized, which had been purified by the "zinc-ammonia" process¹ and which contained 7.87 per cent. of nitrogen, was dissolved in a five-per-cent. solution of acetic acid at room-temperature, a solution of sodium carbonate was added, the flask was repeatedly evacuated and filled with hydrogen and finally allowed to stand overnight. In the morning the well-crystallized precipitate was transferred without exposure to air to a special apparatus in which it was washed with water of 40° C. in an atmosphere of hydrogen. Analysis showed the specimen to contain 8.36 per cent. of nitrogen. Here again we observe an increase in the nitrogen content of our substance.

Again, Fraction II, Specimen II, October, 1904 (N—8.04 per cent.), was twice dissolved in oxalic acid and precipitated from it with ammonia. In the second precipitation the substance was obtained in two fractions of approximately equal weight. The first fraction showed N—7.63 per cent., the second N—7.75 per cent. The two fractions were now combined, crystallized twice from hydrochloric acid (5 per cent.), then three times from a saturated solution of oxalic acid, and finally once more from a five-per-cent. solution of hydrochloric acid. Ammonia was used as the precipitant in all of these processes. The substance now had the following composition: C—58.16 per cent., H—7.07 per cent., N—8.18 per cent.

RECRYSTALLIZATION OF SPECIMENS III AND IV CAUSED NO CHANGE
IN THEIR NITROGEN CONTENT.

In recrystallizing Specimen III, January, 1905 (N—7.34 per cent.), and Specimen IV, January, 1905 (N—7.30. per cent), we

¹ *The Johns Hopkins Hospital Bulletin*, xiii, p. 29, 1902.

chanced to make use of stronger solutions of acids, usually of 15 per cent. strength, and this may possibly account for the fact that we have failed to obtain the marked rise of nitrogen that has just been described. Thus, a part of Specimen III (N—7.34 per cent.) was twice crystallized from a 10-per-cent. solution of hydrochloric acid, ammonia being the precipitant used, and was then found to contain 7.34 per cent. of nitrogen. A second portion of Fraction III was four times precipitated from a 15 per cent. solution of hydrochloric acid, potassium carbonate being the precipitating agent used in each case. After the fourth precipitation the nitrogen content was found to be 7.38 per cent., while a second fraction obtained from the mother liquors after the first precipitation with potassium carbonate had 7.40 per cent. of nitrogen, thus showing that one may obtain a series of fractions,¹ each of which shall have a relatively low nitrogen content.

Specimen IV (N—7.30 to 7.44 per cent.) was twice crystallized from a 15-per-cent. solution of acetic acid, potassium bicarbonate being the precipitant used. After adding the bicarbonate, the solution was made to throw out the base by repeatedly evacuating and allowing hydrogen to enter the flask. Analysis showed 7.47 per cent. of nitrogen. It was then again crystallized from a 10-per-cent. solution of hydrochloric acid, ammonia being the precipitant employed, and was now found to contain N—7.28 per cent. and N—7.36 per cent., C—58.31 per cent., and H—6.96 per cent.

It will be noted, then, that on recrystallizing Specimens III and IV no change took place in the nitrogen content of our substance. We are at a loss to explain why Specimens I and II should have a higher nitrogen content than Specimens III and IV, and also why recrystallization of Specimens I and II should cause the rise in nitrogen there observed. Von Fürth, who also obtained high nitrogen percentages, gives the following table² of his analytical results for material that had been repeatedly recrystallized, nitrogen being determined by the

¹ Bertrand (*Bull. de la Soc. Chim. de Paris*, 1904, p. 1188) lays great stress on the fact that his various fractions of adrenalin showed a nitrogen content of 7.66 per cent. to 7.74 per cent.

² *Monatsh. f. Chem.*, xxiv., p. 261, 1903.

method of Dumas, with the exception of one analysis when the method of Kjeldahl was used:

I				II			
Suprarenin five times crystallized.				Suprarenin six times crystallized.			
C — 58.64 per cent.; 57.99 per cent.				58.72 per cent.; 58.26 per cent.			
H — 7.47	"	"	7.32	"	"	7.21	"
N — 8.43	"	"	8.43	"	"	8.38	"
				(7.65 per cent., Kjeldahl).			

But the single analysis by the method of Kjeldahl must be thrown out of court as faulty, in the face of the four concordant results by the method of Dumas, and this result should not be used in calculating an average percentage of nitrogen, as was done by von Fürth and approved of by Pauly.¹ Von Fürth used dilute hydrochloric acid in recrystallizing his material, but does not state the strength of acid used. It was our opinion at one time that these high nitrogen percentages were caused by retained ammonia, but this assumption, as our own experience has shown, is no longer tenable.

While we have paid attention chiefly to variations in the nitrogen of our substance, we have also made a number of analyses for carbon and hydrogen, some of which have already been given. Put in the form of a table these results are as follows:

From Specimen II. ²			From Specimen IV.	
I	II	III		
C — 58.09 per cent.	58.28 per cent.	58.16 per cent.	58.31 per cent.	
H — 6.96	"	6.89	"	6.96
N — 7.65	"	7.79	"	7.28
				7.36

The percentages here given do not agree sharply with those required by either of the two formulæ now in dispute, the theoretical requirements of which are:

$C_{10}H_{13}NO_3 \frac{1}{2} H_2O$.	$C_9H_{13}NO_3$.
C — 58.82 per cent.	C — 59.02 per cent.
H — 6.86	H — 7.10
N — 6.86	N — 7.65

¹ *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 1388, 1904.

² These data were obtained at different times in the course of the purification of Specimen II, and are not to be taken as the analytical results obtained with one and the same specimen.

We would summarize the results of our work as follows:

In the preparation of epinephrin hydrate by methods that involve the use of hydrogen, specimens obtained at different times showed wide variations in respect to their nitrogen content. Repeated crystallization from dilute solutions of acids apparently induce the nitrogen content of our substance to rise to a point that is not in agreement with any formula hitherto proposed for it. Specimens III and IV, January, 1905, had a nitrogen content of 7.30–7.44 per cent. Perhaps further work would have given us specimens with a still lower nitrogen content, say of 7.00 per cent., as was the case in the specimens described by one ¹ of us two years ago. Those who have prepared our substance only once or twice very naturally entertained no suspicion of the variability in its nitrogen content which we have observed.

ON THE CRYSTALLIZATION OF EPINEPHRIN HYDRATE FROM SOLUTIONS OF POTASSIUM HYDROXIDE.

Having both acid and basic properties our substance is also readily soluble in solutions of the fixed alkalis. From such solutions it is obtained in crystalline form by passing carbon dioxide into them to the point of saturation. In studying the effects on our substance of repeated crystallization from potassium hydroxide solutions we proceeded as follows: A given quantity of material is placed in a round-bottomed flask equipped with appropriate fittings; the air in the flask is removed and hydrogen is introduced in its place. The flask is now immersed in ice-water and a sufficient quantity of an oxygen-free and well-cooled solution of potassium hydroxide (10 to 15 per cent.) is introduced. Solution of the substance is effected in a few moments, and carbon dioxide is then introduced, whereupon complete precipitation occurs. When the precipitate has been thoroughly washed by decantation and also on a filter in an apparatus filled with hydrogen, it is not to be distinguished in any way from specimens that have been crystallized from an acid solution. A slight contamination by potassium silicate is unavoidable, the sphæro-crystals retaining from 0.23 to 0.3 per

¹ Abel, *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 1839, 1903.

cent. of this salt. The losses incurred in repeated crystallizations by this method are no larger than when acids are employed. We lay stress on the fact that the exposure to alkalis in our method is of short duration only. Even under these circumstances a minute quantity of our substance is decomposed by the alkali in each solution, as is proved by the fact that the escaping hydrogen will cause a sensitive solution of litmus to turn blue. But such a decomposition does not affect the results of analysis. That portion of our substance which loses nitrogen at each solution in alkali evidently remains in solution when carbon dioxide is introduced and fails to crystallize out, as otherwise the nitrogen content would steadily decrease with each succeeding solution, whereas, as we shall show, constancy of nitrogen is maintained.

Analytical data:

I. A specimen of one of the fractions described in the preceding section, that contained 7.43 per cent. of nitrogen, was dissolved in the required amount of sulphuric acid of 25 per cent.; a cold 10-per-cent. solution of potassium hydroxide was then introduced until the precipitate that was at first thrown out was again dissolved. Carbon dioxide in excess was then introduced and the flask was placed in ice-water until the crystallization of the base was complete. The crystals consisted of spherical aggregates of minute prisms. The nitrogen content of the substance was found to be 7.21 per cent. and 7.26 per cent.

II. 2.92 grams of one of the fractions obtained during the course of the work on Specimen II, October, 1904, to which reference has been made, which contained 8.00 per cent. of nitrogen, were dissolved in 15 c.c. of a 15-per-cent. solution of potassium hydroxide and precipitated with carbon dioxide. 2.46 grams were recovered with N—7.33 per cent. Of this material 2.26 grams were again treated as before. 1.958 grams were recovered with N—7.10 per cent. 1.735 grams of this fraction were again treated as before, when N was found to be 7.33 per cent.

III. 3.8 grams of repeatedly crystallized material from the same source as that used in the preceding experiment with N—7.62 per cent. were dissolved in 26 c.c. of a 15-per-cent. solution of potassium hydroxide and precipitated as before. Recovered: 3.452 grams with N—7.40 per cent. Of this product 3.13 grams were again dissolved in 20 c.c. of the hydroxide solution. Recovered: 2.834 grams with N—7.12 per cent. Of this material 2.638 grams were again dissolved and precipitated as before, but in the course of filtration in the hydrogen apparatus for the purpose of removing a few fibres of paper, a loss of material occurred, so that only 2.05 grams were recovered. Analyzed for carbon and hydrogen, the results were found to be C—58.69 per cent. and H—7.14 per cent.

8 Epinephrin Hydrate

Hereupon this material that had been thrice crystallized from solutions of potassium hydroxide was dissolved in the hydrogen apparatus at a temperature of 0° C. in a small volume of a ten-per-cent. solution of hydrochloric acid. A cold solution of potassium bicarbonate was now added, and as our base did not at once fall out, the carbon dioxide was removed from the flask and hydrogen introduced, whereupon crystallization took place. Analysis now showed the precipitate to have the following composition:

I	II
C = 58.74 per cent.	58.63 per cent.
H = 7.29 " "	7.11 " "
N = 7.15 " "	7.24 " "

We have seen that the analysis after the 2d and 3d crystallization from potassium hydroxide gave the following results:

C = 58.69 per cent.
H = 7.14 " "
N = 7.12 " "

The theoretical requirements of the formula

$C_{10}H_{13}NO_3 \cdot \frac{1}{2}H_2O$ are:

C = 58.82 per cent.
H = 6.86 " "
N = 6.86 " "

and of the formula $C_9H_{13}NO_3$ are:

C = 59.02 per cent.
H = 7.10 " "
N = 7.65 " "

The analytical results here given certainly speak rather for the correctness of the formula $C_{10}H_{13}NO_3 \cdot \frac{1}{2}H_2O$, than for that of the formula $C_9H_{13}NO_3$, in view of the well-known fact that the method of Dumas uniformly gives percentages of nitrogen which are higher and not lower than those required by theory.

The average of nine nitrogen estimations of specimens which had a high nitrogen content originally, and which were crystallized from potassium hydroxide, is 7.23 per cent. This percentage lies between the requirements of the two formulæ which are under consideration. We have seen that some of the specimens described in the preceding section, which were obtained by the use of acids, also had a nitrogen content considerably below that required by the formula $C_9H_{13}NO_3$. Earlier studies by one¹ of us have shown that dehydration of specimens with a low nitrogen content (7.10–7.20 per cent.) yields a substance which has the composition $C_{10}H_{13}NO_3$.

The monobenzoyl series of our earlier paper also lends support to this formula. The analytical results obtained by Pauly¹ in the case of his urate, and by von Fürth² with his tribenzene-sulpho-derivative do not prove that the formula $C_9H_3NO_3$ is established beyond all doubt. Stolz³ has prepared a trichlorbenzoyl compound by dissolving adrenalin in a dilute solution of sodium hydroxide and then treating it with an excess of p-chlorbenzoyl chloride and a concentrated solution of sodium hydroxide. This compound is a non-crystalline substance of resinous character. The chlorine and nitrogen only of this resin were determined, and while the observed percentages of these elements are in excellent agreement with the theoretical requirements for a trichlorbenzoyl derivative of a substance having the formula $C_9H_3NO_3$, it is to be remembered that this formula is not established with certainty until the percentages for carbon and hydrogen are also found to be concordant with the theoretical requirements.

In view of the various facts that have already been brought forward and the difficulty experienced by all investigators in preparing crystalline and stable derivatives of our substance, we feel justified in believing that the formula $C_{10}H_{13}NO_3 \cdot \frac{1}{2}H_2O$ has not been discredited. The researches of Pauly, Jowett, Stolz, Bertrand, and Abderhalden and Bergell speak unanimously for the correctness of the formula $C_9H_3NO_3$, and it may appear useless under these circumstances to offer renewed objections to its adoption. Our observations, showing that one may obtain specimens with a varying nitrogen content, lead us to believe that if these authors had prepared the substance more frequently and from different lots of glands, they also might have met with this experience, and that in consequence they would have felt less confident of the correctness of their formula. The results obtained by Takamine, Aldrich, and von Fürth, while not in agreement with the requirements of our formula, nevertheless fail to support the formula $C_9H_3NO_3$, and furthermore show that the preparations of two observers may agree in respect to their nitrogen content and at the same time differ

¹ *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 1388, 1904.

² *Monatsh. f. Chem.*, xxiv, p. 261, 1903.

³ *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 4452, 1904.

markedly in their carbon content. When it is held in mind that we have no means of purifying our substance by crystallization from a solvent, and that we must depend for its preparation solely on precipitation from gland extracts with ammonia and other alkalies, it will at once be apparent why the present writers attach so much importance to the finding of specimens with a low nitrogen content. We hold that such specimens are least contaminated with products of a high nitrogen content such as the gland is known to contain.

We would also state that the half molecule of water ($\frac{1}{2}\text{H}_2\text{O}$) of our empirical formula has always been regarded by us to be water of constitution. The molecular weight of the substance must therefore be expressed by at least $2(\text{C}_{10}\text{H}_{13}\text{NO}_3\frac{1}{2}\text{H}_2\text{O})$. The molecular-weight determinations of von Fürth,¹ Jowett,² and Bertrand³ appear to invalidate such an assumption, inasmuch as these writers obtain values that are in agreement with a molecular weight which is only half as large as that here assumed. Von Fürth's determinations by the freezing-point method were made with an impure tribenzenesulpho-derivative and are not conclusive. His analyses show that his percentages for carbon are 0.74 per cent., for sulphur, 1.45 per cent. below the theoretical requirements of his formula. It is not improbable that he was dealing with a mixture of di- and tribenzenesulpho-derivatives. Jowett and Bertrand used glacial acetic acid as a solvent for our substance in determining its molecular weight, a procedure that is entirely inadmissible, since the substance can not again be recovered unchanged after it has been subjected to the manipulations with this solvent that are required in making the determinations. This very important point, which has been apparently overlooked by Jowett and Bertrand, seems to us to deprive their results of the confirmatory value which they attach to them.

AMMONIA, A DECOMPOSITION PRODUCT OF EPINEPHRIN HYDRATE.

In the presence of air and moisture, epinephrin hydrate slowly undergoes spontaneous decomposition with evolution of

¹ *Monatsh. f. Chem.*, xxiv, p. 261, 1903.

² *Trans. Chem. Soc.*, 1904, p. 194.

³ *Bull. de la Soc. Chim. de Paris*, xxxi, p. 1188, 1904.

volatile bases. When it is treated with boiling water in a current of pure hydrogen, ammonia and methylamine and possibly other bases are liberated. The following experiments will illustrate our method of isolating these bases.

4.49 grams of an ash-free specimen, several times crystallized, with a nitrogen content of 7.74 per cent., were placed in a liter flask of Jena glass that was then freed of air and filled with hydrogen. Hereupon 500 c.c. of water that had been boiled in a current of hydrogen were introduced. The flask was now attached to a condenser and immersed in an oil bath at a temperature which just sufficed to keep the contents of the flask at the boiling-point. A rapid current of washed hydrogen was allowed to pass through the boiling mixture during the whole experiment. A small quantity of dilute hydrochloric acid was placed in the receiver and the distillation carried on until about 420 c.c. of distillate were obtained. Under exclusion of air, about 400 c.c. of oxygen-free water were again introduced into the distilling flask, and this was then allowed to stand under hydrogen pressure from Saturday evening until the following Monday morning, when the distillation was proceeded with as before. The flask was then charged as before, its contents were again concentrated to about 80 c.c., and this process was repeated until more than two liters of distillate were obtained. The combined distillates were now concentrated until the chlorides present began to crystallize. At this point a separation into two fractions was attempted by means of a small quantity of absolute alcohol. The chlorides that did not at once dissolve in alcohol yielded 0.2039 gram of chlorplatinate (Fraction I), and these were found to consist of octahedral crystals like those of ammonium chlorplatinate and six-sided plates characteristic of methylamine chlorplatinate.

The alcoholic solution yielded 0.5630 gram of chlorplatinate (Fraction II). This fraction was dissolved in from 25 to 30 c.c. of hot water, the solution somewhat concentrated and set aside to crystallize. The crystals obtained weighed 0.1762 gram (Fraction IIa), and were found to consist of octahedra and six-sided plates with some pointed prisms.

It is seen, therefore, that a total quantity of 0.7669 gram of mixed chlorplatinate was obtained in the above experiment

in which 4.49 grams of our substance were boiled with distilled water alone.

Analysis: Fraction I. 0.2037 gram of Pt salt gave		
0.0851 gram Pt	Pt therefore	=41.77 per cent.
Methylamine chlorplatinate, $(\text{NH}_2\text{CH}_3)_2 \text{H}_2\text{PtCl}_6$		
		=41.28 per cent. Pt required.
Ammonium chlorplatinate, $(\text{NH}_3)_2 \text{H}_2\text{PtCl}_6$		
		=43.80 per cent. Pt required.

Fraction IIa. 0.1762 gram of Pt salt gave		
0.0734 gram Pt	Pt therefore	=41.66 per cent.

A mixture of four parts of methylamine chlorplatinate and one part of ammonium chlorplatinate requires Pt—41.80 per cent. We have ocular proof of the presence of the latter salt in the products above analyzed; it may therefore be assumed that Fraction I contained 20 per cent of ammonium chlorplatinate. The presence of di- or trimethyl ammonium chlorplatinate is excluded, since admixture with these salts would give a much lower platinum content than was found above. We are confident of the correctness of our assumption that the octahedral crystals described by us as present in such large proportion were in reality crystals of ammonium chlorplatinate. In a trial distillation with 1.34 grams of epinephrin hydrate, which preceded the experiment above described, we found our first fraction of chlorplatينات to consist almost entirely of these octahedra. This fraction weighed only 0.0151 gram and contained 42.38 per cent. Pt. While we cannot claim that this analysis is of equal value with those given above, the high platinum percentage obtained is nevertheless in agreement with the observed fact that the chlorplatينات in question consisted largely of octahedral crystals.

The filtrate and washing from Fraction IIa, as above described, were treated with hydrogen sulphide, the platinum-free filtrate was concentrated and tested for methylhydrazine with negative results. The high solubility in water of this portion of our mixed chlorplatينات leads us to suspect that ammonia and methylamine are not the only volatile bases that are liberated when epinephrin hydrate is boiled with water in a current of hydrogen.

After discontinuing the boiling of our 4.49 grams of epinephrin hydrate with distilled water as above described, the process of distillation was again taken up, but this time 110 c.c. of a 33-per-cent. solution of potassium hydroxide were introduced together with 200 c.c. or more of water. When the distilling flask needed refilling, distilled water was introduced. The distillation was carried on until the distillate was no longer alkaline to litmus paper. The receiver in this case was charged with dilute sulphuric acid. The combined distillates were collected and concentrated and the final product when nearly dry was treated with absolute alcohol. Only a very small quantity of sulphate remained undissolved by the alcohol and this yielded chlorplatinate consisting largely of octahedral crystals. The alcohol-soluble sulphate yielded 0.660 gram of mixed chlorplatinate. Here again many octahedral crystals were present among the six-sided plates of methylamine chlorplatinate. 0.2627 gram of Pt salt yielded 0.1096 gram Pt. Therefore $\text{Pt} = 41.72$ per cent. Here again with octahedral crystals present in abundance we may assume that ammonium chlorplatinate was present to the extent of 20 per cent.

DECOMPOSITION PRODUCTS OF THE BASE, $\text{C}_3\text{H}_4\text{N}_2\text{O}$, OBTAINED
BOTH FROM EPINEPHRIN, $\text{C}_{10}\text{H}_{13}\text{NO}_3$, AND ITS HY-
DRATE, $\text{C}_{10}\text{H}_{13}\text{NO}_3 \cdot \frac{1}{2}\text{H}_2\text{O}$.

The base, $\text{C}_3\text{H}_4\text{N}_2\text{O}$, has been described in detail in an earlier paper,¹ in which it was suggested that this is a highly unstable cyclic compound closely related to substances of the pyrazolon series. As the base itself is highly unstable and has not been obtained in the free state, its stable gold salt, $\text{C}_3\text{H}_4\text{N}_2\text{O} \cdot \text{HCl} \cdot \text{AuCl}_3$, was used in the study of its decomposition products.

Five grams of the well-crystallized gold salt were introduced into a Kjeldahl flask, 20 c.c. of a 4-per-cent. solution of potassium hydroxide were added, and then 60 c.c. of a 50-per-cent. solution of the same alkali were introduced, the flask and solutions being well cooled. A previous attempt to mix the gold salt with powdered potassium hydroxide had taught us to observe these precautions, as in this case the heat of reaction

¹ *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 368, 1904.

caused the mixture to puff up with explosive violence. The contents of the Kjeldahl flask, as thus prepared, were now subjected to distillation, the distillate being received in a well-cooled receiver containing 20 c.c. of N/5 sulphuric acid. The distillate is strongly alkaline from the very first, but does not reduce Fehling's solution or ammoniacal silver nitrate solution until about one-third of the fluid in the distilling flask has passed over into the receiver. At this point the distillate begins to reduce Fehling's solution with great power and has the odor of methylhydrazine.

At the close of the experiment the contents of the receiver were found to be alkaline, the 20 c.c. of N/5 sulphuric acid that had been placed in it being insufficient to neutralize the basic products evolved from the gold salt, $C_3H_4N_2O \cdot HCl \cdot AuCl_3$.

In order to isolate the reducing substance, the alkaline distillate containing it was shaken with a small quantity of benzaldehyde and allowed to stand for twelve hours, when it was found that yellowish aggregates of an apparently crystalline compound had formed. The manner in which this compound was formed, its solubility in alcohol and ether, and its behavior in the operations described below compel us to believe that it was tribenzalmethylhydrazine,¹ $(C_6H_5 \cdot CH)_3(N_2CH_3)_2$.

The presence of an excess of benzaldehyde gave to the distillate the character of an emulsion and prevented all of the hydrazine compound from separating. The entire mixture was therefore shaken with ether, which removed the benzalmethylhydrazine and the excess of benzaldehyde, as also some of the uncombined ammonia and methylamine. To the ethereal solution a few drops of sulphuric acid and a little alcohol were added, the ether was evaporated on the water-bath, more alcohol was added, and the solution was now concentrated until the benzalmethylhydrazine was judged to be decomposed. The residue was now treated with absolute alcohol, whereupon a small quantity of salt, consisting mainly of ammonium sulphate, was thrown out. The chlorplatinate of this crystalline sulphate was analyzed: 0.2224 gram Pt salt yielded 0.0961 gram Pt. Therefore Pt = 43.21 per cent. The crystals were chiefly

¹ C. Harries and T. Haga. *Ber. d. deutsch. chem. Gesellsch.*, xxxi, p. 61, 1898.

octahedra with only here and there a six-sided plate. As ammonium chlorplatinate, $(\text{NH}_4)_2\text{H}_2\text{PtCl}_6$, requires 43.90 per cent. Pt, it will be seen that our platinum salt consisted mainly of ammonium chlorplatinate.

The alcoholic filtrate from the ammonium sulphate was now concentrated until slender prisms began to appear. These had the appearance and solubilities and gave all the reactions characteristic of the acid sulphate of methylhydrazine. Methylhydrazine and its salts were prepared by us, and the identity of this decomposition product with methylhydrazine was established by comparative tests. The crystals of the acid sulphate, to which reference has just been made, were recrystallized from hot alcohol, when their melting-point was found to be 139° to 140° C., while the melting-point of the acid sulphate of methylhydrazine is given as 139.5° C. by von Brüning.¹

This fact, taken with the power of the salt to reduce Fehling's solution at room temperature, to give a mirror with silver nitrate, and to form an ether-soluble and crystalline compound with benzaldehyde, not to mention the characteristic odor of the free base, proves beyond doubt that methylhydrazine is one of the decomposition products of the base $\text{C}_3\text{H}_4\text{N}_2\text{O}$. It may also be mentioned that on fusing the crystals of our acid sulphate with a pellet of potassium we obtained clear proof of the formation of potassium cyanide, thus showing that this powerfully reducing salt contains both carbon and nitrogen.

The fluid from which the benzalmethylhydrazine was separated by means of ether was distilled with an excess of pure sodium hydroxide, and the volatile bases that were thus obtained were identified by means of platinic chloride. Fraction I of the platinum salts, recrystallized from hot water, was found to consist only of octahedral crystals. 0.1853 gram of this Pt salt gave 0.0812 gram Pt. Therefore, Pt = 43.82 per cent. Ammonium chlorplatinate, $(\text{NH}_4)_2\text{H}_2\text{PtCl}_6$, requires Pt — 43.90 per cent. This portion, therefore, consists entirely of ammonium chlorplatinate. Fraction II of the platinum salts consisted largely of the characteristic six-sided plates of methylamine chlorplatinate. 0.2538 gram Pt salt gave 0.1017 gram

¹ *Ann. d. Chem.*, ccliii, p. 5, 1889.

Pt. Therefore Pt = 40.07 per cent. Methylamine chlorplatinate, $(\text{CH}_3\text{NH}_2)_2\text{H}_2\text{Pt Cl}_6$, requires Pt — 41.30 per cent. Dimethylamine chlorplatinate, $((\text{CH}_3)_2\text{NH})_2\text{H}_2\text{PtCl}_6$, requires Pt — 38.98 per cent. We can not undertake to say just which chlorplatinate of lower platinum content was here mixed with the six-sided plates of the methylamine salt, but we nevertheless feel confident that this latter salt was present to the extent of at least 50 per cent.

We have now proved that ammonia and methylhydrazine are degradation products of our base, $\text{C}_3\text{H}_4\text{N}_2\text{O}$, but we are also reasonably certain that methylamine is to be included with them.

SKATOL, PROTOCATECHUIC ALDEHYDE, AND VANILLIN AS DECOMPOSITION PRODUCTS OF EPINEPHRIN HYDRATE.

In an earlier paper ¹ it was shown that skatol is formed when the monobenzoyl compound of epinephrin is fused with a fixed alkali. While this decomposition product is of secondary importance only among the degradation products of our substance, it may be deserving of mention that it is also to be obtained from epinephrin hydrate itself. We have observed that whenever a quantity of this substance is heated in a Kjeldahl flask with a concentrated solution of potassium hydroxide until most of the water has been expelled and the flask is on the point of fusing, the odor of skatol at once becomes very pronounced in the distillate. However, a much better yield of skatol is obtained by the following process: 1.5 grams of epinephrin hydrate are dissolved in a very little dilute hydrochloric acid, an excess of a strong solution of platinic chloride is added, the flask is attached to a reflux condenser, and its contents are boiled for two hours. The reduced platinum is separated by filtration, while that remaining in solution is removed by hydrogen sulphide. The filtrate is concentrated, and the excess of hydrochloric acid is expelled from it by repeatedly evaporating it with absolute alcohol. A hygroscopic residue which crystallizes in part and which still reduces Fehling's solution is thus obtained. It is evident we are dealing here with a partially oxidized epinephrin hydrate. If this residue be now heated in

¹ *Zeitschr. f. physiol. Chem.*, xxviii, p. 345, 1899.

a flask with a 40 per cent. solution of potassium hydroxide until the water of the solution has been almost entirely removed, then acidulated with sulphuric acid and shaken with ether, a considerable quantity of skatol may be obtained. With only a part of the residue resulting from the 1.5 grams of epinephrin hydrate that were used in the above experiment, we obtained in this way enough skatol for the nitrous acid reaction and for the color reaction with concentrated hydrochloric acid.

Vanillin: Stolz¹ has stated that when epinephrin hydrate (adrenalin) is treated with methyl iodide and a solution of sodium hydroxide in methyl alcohol, an incomplete methylation takes place, with the result that vanillin is formed. In the absence of any statement to the contrary by Stolz, and in view of the fact that only very small amounts of the closely related protocatechuic acid have been obtained from adrenalin, we may assume that the yield of vanillin obtained by Stolz was likewise very inconsiderable. Several months before the appearance of this paper by Stolz we also obtained small quantities of vanillin, but under very different experimental conditions, and of late we have frequently verified our earlier observations. In these early experiments it was repeatedly observed that when epinephrin hydrate is oxidized with lead peroxide or manganese dioxide, or with such a quantity of sodium hypobromite that three atoms of oxygen are liberated for each molecule of epinephrin hydrate, an ether-soluble substance is obtained which, either immediately or on standing exposed to the air for a day or two, gives rise to the odor of vanillin. When this substance was carefully oxidized with ferric chloride or with an alkaline solution of potassium permanganate, a larger amount of vanillin was produced, so that it could be extracted and identified both by its odor and by its characteristic behavior with phloroglucin and concentrated hydrochloric acid. Later we obtained the odor of vanillin on boiling epinephrin hydrate in a test-tube with solutions of sodium sulphite and ferric chloride. The following method also was found to yield a small quantity of vanillin: 1 gram of chloroform and 4 grams of sodium hydrate dissolved in 8 c.c. of water were added to 1.5 grams of epinephrin hydrate, the mixture was heated to the boiling-point

¹ *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 4150, 1904.

of the chloroform, then set aside, again heated, and then set aside for twelve hours. The solution was then repeatedly extracted with ether, the residue obtained on evaporating the ether was extracted with warm benzene, and the benzene solution was allowed to evaporate at room temperature. The partially crystalline residue had the odor of vanillin and gave the phloroglucin-vanilleïn reaction in a manner not to be distinguished from that which follows the use of vanillin itself.

Protocatechuic Aldehyde and its methyl ether, Vanillin: We have lately made some improvements in that one of the above methods which involves the use of sodium hypobromite. In each of four recent trials with this method we have obtained protocatechuic aldehyde in considerable amounts and also vanillin in quantities that suffice for its identification. Success depends here on the observance of three precautions, namely, that the oxidation be carried on rapidly, that the temperature be not allowed to rise above 60°C ., and that such a quantity of hypobromite solution be used as shall only partially oxidize the base. To cite an example: 4 grams of epinephrin hydrate were dissolved in 20 c.c. of a 20 per cent. solution of sodium hydroxide, and a freshly prepared hypobromite solution (9.3 grams of bromine in 40 c.c. of a 20 per cent. solution of sodium hydroxide) was immediately added in small quantities at a time, the solution being vigorously shaken meanwhile and not allowed to rise above 60°C . The operation, which requires perhaps fifteen minutes, is finished when acidulation of a portion of the solution, after the last addition of hypobromite, fails to liberate bromine. The solution is now neutralized at 0°C . with dilute sulphuric acid, then made plainly acid, filtered from pigmentary substances which have been thrown out, and extracted four times with ether recently purified and free from peroxides. The residue obtained on evaporating the ether is repeatedly extracted with small quantities of warm benzene. The benzene solution in a few moments deposits glittering, star-shaped clusters of prismatic crystals, and continues to do so as the benzene is poured off and allowed to stand.

This crystalline substance is not to be confounded with either protocatechuic acid or with pyrocatechin, since the first of these two substances is practically insoluble in boiling benzene, and

the second is readily soluble in cold benzene. In order more effectually to exclude protocathechuic acid, the first crop of crystals was rejected and only the later crops of well-formed colorless crystals were recrystallized from warm benzene. It was found that three successive yields of crystals obtained by recrystallization, all showed a melting-point of 149° – 150° C. Even the second deposit of colorless crystals obtained from the first benzene extract of the ether residue had this melting-point. This constancy of the melting-point would indicate that we are dealing here with a chemical individual and not with a mixture of substances. The substance is fairly soluble in cold water, readily soluble in alcohol and ether; its aqueous solutions assume a canary-yellow color on the addition of alkalis, give the characteristic protocathechuic reaction with ferric chloride, reduce ammoniacal solutions of silver nitrate at room temperature, do not reduce Fehling's solution on boiling, and give a light-yellow flocculent precipitate with lead acetate. Mixed with an excess of phloroglucin and dissolved in a few drops of absolute alcohol, it will assume a deep-red color on the addition of a few drops of concentrated hydrochloric acid. The reaction in this case is, however, easily differentiated from the phloroglucin-vanillein reaction. The acidulated alcoholic solution never deposits a sediment, nor is it rendered turbid by the further addition of hydrochloric acid; the red color fades quickly when more acid is added and the addition of a few drops of water at a time when it is most intense causes it to disappear instantly. When the reaction is made in the same way with similar quantities of vanillin in place of our substance, the difference is striking. In this case the addition of concentrated hydrochloric acid causes a deposition of what is presumably red phloroglucin-vanillein, and the whole mixture retains its bright cherry-red color on the addition of water, but on standing for an hour or two such a diluted mixture will separate into a colorless solution and a bright-red granular sediment. We lay stress on the fact that the vanillin obtained by us from epinephrin hydrate and which, as will presently appear, passes into benzene in company with the above crystals, gives the phloroglucin reaction in the manner just described.

We believe there can be no doubt in respect to the identity of

the crystalline substance which we have just described. Potocatechuic aldehyde, whose melting-point is given as 150° C. by Fittig and Remsen,¹ has the same solubility in all of the solvents named, and behaves in an identical manner toward every reagent which we have employed. The acid which corresponds to this aldehyde, namely, protocatechuic acid, has hitherto been obtained in small amounts only by fusing epinephrin hydrate with one of the fixed alkalies, a method which is open to criticism on the ground that a substance containing the vanillic or similar residues will also yield protocatechuic acid when treated in this way. We would state in this connection that we have thus far made but one experiment on the behavior of vanillin toward solutions of sodium hypobromite, and have found that a small quantity remains unaltered, while most of it is changed into a substance with the following properties: It crystallizes from benzene, is insoluble in water, only very slightly soluble in alcohol. Its alcoholic solutions assume a steel-blue color on the addition of a very small quantity of ferric chloride, and this color passes at once into a shade of brownish-green on the addition of an excess of this reagent. Recrystallized, the substance melted at 162° C. We have not yet isolated the other products, if there be such, that result from this treatment of vanillin, and we have referred to the experiment for the purpose of showing that some vanillin may remain unaltered in the treatment to which epinephrin hydrate was subjected.

As has been stated, vanillin is always obtained in small amount in association with protocatechuic aldehyde by the method of oxidation just described. The bowls in which the first benzene solutions are allowed to deposit their protocatechuic aldehyde emit the odor of vanillin after pouring off the solvent. The final residue, which is obtained from the benzene after repeatedly pouring it off from the crystallized aldehyde, gives this odor still more markedly. On carefully oxidizing such a residue with a very dilute alkaline solution of potassium permanganate, acidulating with dilute sulphuric acid, and shaking out with ether, the partially crystalline product was pro-

¹ *Annal. d. Chem.*, clix, p. 148, 1871. Wegscheider, *Monatsh. f. Chem.* xiv, p. 383, finds the melting-point to be 153° – 154° C

nounced by several persons to have the odor of vanillin. . This product also behaved toward phloroglucin exactly as does synthetic vanillin. Absorbed as we were in our attempt to separate vanillic alcohol from the products of the oxidation with sodium hypobromite, we neglected to use a bisulphite solution in the isolation of our vanillin. Stolz¹ relied on the crystalline character of his substance, on its odor, and on its behavior in ether toward a bisulphite solution for its identification as vanillin. In place of the bisulphite test we must offer the phloroglucin-vanillein reaction, a reaction which, in company with the ferric-chloride test, is not given by any other substance whose odor could be mistaken for that of vanillin. We therefore agree with Stolz in believing that this substance is obtainable from epinephrin hydrate, but we attach much more importance to its appearance than does this author. He accounts for it on the assumption that in his experiments with methyl iodide and sodium hydrate it was produced in consequence of the partial methylation of a protocatechuic residue. Even under such conditions a part of the vanillin obtained may owe its origin to a preformed vanillic complex. Until our results shall be otherwise explained, we must hold to the opinion that epinephrin hydrate contains a vanillic residue, $C_6H_3(OH)-(O.CH_3).C$. Two other observations strengthen us in this conclusion. More than a year ago, in beginning a systematic study of the behavior of epinephrin hydrate toward the whole list of oxidizing agents in use in the laboratory, we several times obtained a small quantity of an oily substance which had the odor of guaiacol, and recently we have observed that epinephrin hydrate gives a color-reaction with phloroglucin which is very like that given by vanillin. If equal quantities of these two substances be intimately mixed, placed in a porcelain bowl and moistened with a single drop of hydrochloric acid, a pink color is developed, the addition of a second drop intensifies the color, and on allowing the material to flow over the surface, the bowl appears to have a cherry-red lining. In this connection it may also be stated that the crystals of protocatechuic aldehyde which are deposited from the first benzene extracts in the above oxidation experiments and which have the odor of vanillin

¹ *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 4151, 1904.

because of contamination with it or with vanillic alcohol, give, when dissolved in concentrated sulphuric acid, a color reaction like that given by vanillic alcohol.

Had we obtained vanillin only by the action of sodium hypobromite on epinephrin we should be inclined perhaps to attach little significance to its appearance. It might be urged that methyl bromide is formed as the first step in the use of this reagent, and that the bromide in the presence of the alkali acts as a methylating agent upon the protocatechuic residue, thus producing vanillin; or, that commercial bromine frequently contains a little methyl bromide, in which case the argument just advanced would also apply. When it is recalled, however, in how many ways vanillin has been obtained by us it will be seen how untenable are such assumptions. No charge of direct methylation can be brought against these other methods. It therefore only remains for our critics to advance the hypothesis *that in all these instances a methyl group was shifted from the side chain to the hydroxylated benzene nucleus*. We are not aware that a transfer of this kind has ever been observed to take place in the use of oxidizing agents of only medium power, and also in the use of a fixed alkali and of chloroform as in the experiments cited above.

Our observations in respect to the occurrence of vanillin among the degradation products of epinephrin hydrate are not in harmony with the results of von Fürth,¹ who failed to obtain any evidence of the existence of a methoxy group in suprarenin on treating it with hydriodic acid by Zeisel's method. We are aware that Hewitt and Moore² have found that vanillin yields the theoretical amount of O-methyl when it is tested by this method. Vanillin in combination with other residues need not, however, give off its methyl group with equal ease. Von Fürth states briefly that suprarenin contains no methoxy group, but he furnishes no data such as the length of time during which the substance was tested, or whether it was resinified by the concentrated hydriodic acid, points which now become of importance. The literature of chemistry contains numerous

¹ *Monatsh. f. Chem.*, xxiv, p. 263, 1903.

² A Modification of Zeisel's Method for the Estimation of Methoxy Groups. *Jour. Chem. Soc. Trans.*, lxxxix, pp. 318-321.

examples of inadequate results given by this method. Decker and Solinina¹ found that only one of the two ethoxy groups contained in a certain nitrosophenol derivative was removed by the use of Zeisel's method, while the other was liberated only under the conditions which obtain in the method of Herzig and Meyer. Later, Decker² reported that only prolonged boiling (three to four hours) with saturated hydriodic acid enabled him to obtain the required amount of O-ethyl in the case of several bodies. Inadequate, but far from negative, results have also been obtained in the case of substances containing both O- and N-methyl,³ as well as in the case of such as contained only alkyloxy groups.⁴ Our results certainly justify us in raising the question whether epinephrin hydrate may not belong to that class of substances whose alkyloxy groups are not easily severed from the molecule. Such resistant groups may be wrongfully estimated along with concomitant N-alkyl groups unless suspicion is aroused, as in our case, by evidence otherwise obtained, that the reaction has proceeded in an anomalous manner.

On treating his substance by the method of Herzig and Meyer, von Fürth obtained methyl iodide, but in such amount only as indicated 4.79 per cent. and 5.19 per cent. of N-methyl, whereas his formula, $C_9H_{13}NO_3$, requires the presence of 8.2 per cent. of N-methyl on his assumption that it contains the methylimide ($:N.CH_3$) group. If we assume that epinephrin hydrate, $2(C_9H_{13}NO_3 \cdot \frac{1}{2}H_2O)$, contains a methyl group linked to one of its two nitrogen atoms and that a second methyl group is attached to oxygen, it will follow that the calculated yield for both kinds of methyl is 7.35 per cent., while that for each alone is 3.76 per cent. Von Fürth's results agree fully as well with our assumption as with his. The appearance of vanillin in our experiments demands that epinephrin hydrate

¹ Ber. d. deutsch. chem. Gesellsch., xxxv, p. 3221, 1902.

² Ber. d. deutsch. chem. Gesellsch., xxxvi, p. 2895, 1903. See also O. Hesse, Ber. d. deutsch. chem. Gesellsch., xxx, p. 1985, 1897; and Bistrzycki and Herbst, Ber. d. deutsch. chem. Gesellsch., xxxv, p. 3140, 1902.

³ Freund, Ber. d. deutsch. chem. Gesellsch., xxxii, p. 183, 1899.

⁴ Moldauer, Monatsh. f. Chem., xvii, p. 470, 1896. Bamberger, Monatsh. f. Chem., xv, pp. 509-510.

should be again tested for the presence of an oxymethyl group by the method of Zeisel. Prolonged boiling with saturated hydriodic acid, followed by treatment according to Herzig and Meyer, might lead to results that would agree with our observations and leave no doubt that one of the two benzene nuclei in epinephrin hydrate consists of a vanillin residue $\text{C}_6\text{H}_3(\text{OH})(\text{O}-\text{CH}_3)\cdot\text{C}$. In view of the fact that the base also yields protocatechuic acid and that after methylation and subsequent oxidation it yields veratric acid,¹ the second benzene nucleus must exist in the form of the protocatechuic complex, $\text{C}_6\text{H}_3(\text{OH})_2\cdot\text{C}$.

We have seen that a considerable quantity of methylamine is liberated when epinephrin hydrate is boiled with water in a current of hydrogen. Pauly, Jowett, and Stolz have all obtained small quantities of this volatile base on treating their material with solutions of the fixed alkalis, or on oxidizing it with potassium permanganate (Jowett). This result taken with other considerations has led these authors to believe that epinephrin hydrate contains the group $\cdot\text{NH}\cdot\text{CH}_3$ attached to a side chain, in place of the $\text{:N}\cdot\text{CH}_3$ group assumed by von Fürth. Our own observations force us also to accept the conclusion that one of the two nitrogen atoms of epinephrin hydrate is present in the complex $\text{NH}(\text{CH}_3)$. The other nitrogen atom of our double molecule, which like the first must exist in a side chain, we assume to be present in the group :NH . We offer this suggestion in explanation of the appearance of ammonia when epinephrin hydrate is boiled with water, and also because no evidence is obtainable to indicate the presence of a primary amine residue, while Pauly has shown that epinephrin hydrate behaves toward phenyl mustard oil like a secondary amine, although a nitrosamine was not obtainable.

We venture on no hypothesis of our own in respect to the disposition of the hydroxyl groups which we must assume to be present outside of the benzene nuclei; in respect to the manner in which the intramolecular dehydration occurs, when the hydrate, $2(\text{C}_{10}\text{H}_{13}\text{NO}_3\frac{1}{2}\text{H}_2\text{O})$, is changed into the alkaloidal

¹ Jowett, *Trans. Chem. Soc.*, lxxxv, pp. 195-196. Stolz, *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 4149, 1904.

body, $2(\text{C}_{10}\text{H}_{13}\text{NO}_3)$, nor as to the manner in which the two side chains, so to speak, of our molecule are connected.

OBJECTIONS TO THE FORMULA, $\text{C}_6\text{H}_5(\text{OH})_2\text{CH.OH.CH}_2\text{.NH.CH}_3$, NOW GENERALLY ACCEPTED AS MOST PROBABLY REPRESENTING THE CONSTITUTION OF OUR SUBSTANCE.¹

1. The analytical data which we have given in a preceding section of this paper, together with the arguments there advanced, justify us in contending that the elementary formula, $\text{C}_9\text{H}_{13}\text{NO}_3$, is not established with that certainty which is a prerequisite to a study of the chemical constitution of a compound.

2. We have shown in an earlier paper² that epinephrin hydrate, $2(\text{C}_{10}\text{H}_{13}\text{NO}_3\frac{1}{2}\text{H}_2\text{O})$, is readily dehydrated and thereby changed to the alkaloidal base, $2(\text{C}_{10}\text{H}_{13}\text{NO}_3)$. It is this observation especially which has forced us to doubt the validity of the molecular-weight determinations which have appeared to corroborate the formula $\text{C}_9\text{H}_{13}\text{NO}_3$. To those who wish to repeat this experiment, we would say that the dehydration must be effected at as low a temperature as possible. During the past year we have observed that a concentrated solution of trichloroacetic acid (1:1) also effects this dehydration, but we have not determined whether, in addition to the loss of a molecule of water, other changes may not here take place.

In an earlier paper it was also shown that the monobenzoyl epinephrin (dehydrated) is capable of taking up three acetyl groups. The dehydrated molecule is therefore able to bind four acidyl groups in proportion to ten atoms of carbon (or nine if the formula of Aldrich be correct). This capacity of dehydrated epinephrin for holding acid radicles can not be made to harmonize with the views now held in respect to its constitution. It is difficult to see how a substance of the assumed

¹ See Jowett, *Trans. Chem. Society*, 1904, p. 192. Stolz, *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 4149, 1904. Pauly, *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 2944, 1903, and xxxvii, p. 1388, 1904. Friedmann, *Beitr. chem. Physiol. u. Pathol.*, vi, p. 92, 1904.

² *Ber. d. deutsch. chem. Gesellsch.*, xxxvi, p. 1844, 1903.

constitution, $C_6H_3(OH)_2CH.OH.CH_2.NH.CH_3$, can lose the elements of water and still retain this property of binding four acid radicles.

3. It has been shown that when our substance is boiled with water in an atmosphere of hydrogen, both methylamine and ammonia are liberated. Now a substance with the chemical structure above assumed would hardly be expected to yield both of these bases under these conditions. Apparently such a substance, *if decomposed at all by this treatment*, would yield methylamine alone and not ammonia also.

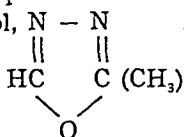
4. The appearance of vanillin which has been repeatedly found by us to be present among the degradation products of epinephrin hydrate is also not explainable if we accept the structural formula $C_6H_3(OH)_2.CH.OH.CH_2.NH.CH_3$.

5. The advocates of this structural formula have also failed to show that a substance of the character assumed can yield the peculiar and highly unstable base, $C_3H_4N_2O$, which is so easily obtainable on oxidizing either epinephrin or its hydrate with nitric acid. It may be incorrectly surmised in some quarters that the discovery of this base can throw no light on the constitution of epinephrin, since possibly it is synthetically produced in the course of the oxidation, being itself the result of an interaction between the side chain of epinephrin and nitric acid. On such an hypothesis the nitrogen of the base, $C_3H_4N_2O$, would owe its origin entirely or in part to the nitric acid employed, and the base would be formed in much the same way as glyoxalin, $C_3H_4N_2$, appears, when ethyl alcohol is oxidized with nitric acid. We cannot admit that such an assumption, however plausible in appearance, has any foundation in fact. It has already been stated ¹ that the base is still obtainable after epinephrin has been subjected to a preliminary oxidation with chromic acid, a procedure which would apparently either destroy or so modify the side chain of our molecule as to render it incapable of interacting with nitric acid in the manner assumed. The fact that this base, $C_3H_4N_2O$, yields ammonia and methylamine among its degradation products in like manner with epinephrin, also encourages us in the belief that all of its nitrogen

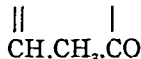
¹ Ber. d. deutsch. chem. Gesellsch., xxxvii, p. 377, 1904.

is derived from epinephrin and that it is to be reckoned with in any study of the constitution of this substance.

We have shown conclusively that this singular base yields methylhydrazine, $\text{CH}_3\text{NH.NH}_2$, on treatment with alkalis, a fact which forces us to believe that its two nitrogen atoms are directly linked the one to the other, and that it has the chemical structure of an open chain or cyclic hydrazide. Such experiments as we have made with a view to deciding this point, while not conclusive in character, rather induce us to believe that the base is a new and highly unstable cyclic hydrazide. If it contains a methyl group preformed, it might reasonably be supposed to possess a structure something on the order of a methyl oxybiazol, $\text{N} - \text{N}$, while if the methyl group of its de-



composition products is secondarily produced from a methylene group in the course of the treatment with alkalis, a structural formula on the order of the pyrazolon series, $\text{N} - \text{NH}$, would be more in accord with the facts observed.

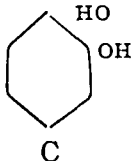


be more in accord with the facts observed.

We are far from asserting that this substance, $\text{C}_3\text{H}_4\text{N}_2\text{O}$, even though we believe that its two nitrogen atoms are derived from epinephrin, exists as such in the epinephrin molecule. Assuming for the moment that the groups $:\text{NH}$ and $.\text{NH.CH}_3$ are present in the side chain of the molecule $2(\text{C}_{10}\text{H}_{13}\text{NO}_3 \cdot \frac{1}{2}\text{H}_2\text{O})$, it is conceivable that in the production of the base, $\text{C}_3\text{H}_4\text{N}_2\text{O}$, ring closure took place in such a way that the nitrogen atoms of these two disconnected groups became directly joined. The appearance of methylhydrazine among the decomposition products of the base has very naturally induced us to search for hydrazine and methylhydrazine among the degradation products of epinephrin hydrate itself, but thus far our search has not met with success.

We venture to think that the foregoing objections speak with some force against the validity of the formula $\text{C}_6\text{H}_3(\text{OH})_2\text{CH.OH.CH}_2.\text{NH.CH}_3$, as correctly representing the structure of

our substance, and we offer them with no desire to defend our past work at all costs and with no intention of disparaging the work of our opponents in the study of this interesting compound. We admit the force of the arguments which speak for the presence of a protocatechuic residue, but we are forced by our own work to point out that the vanillin complex, $C_6H_3(OH).(O.CH_3)$ (1.2.), may also be found to be present. Further experiments as to the constitution of the nitrogenous complex or side chain with which these nuclei are associated appear to us to be necessary before a definite conclusion can be announced in respect at least to the location of its hydroxyl groups. In spite of the considerable success with which present-day efforts¹ have been rewarded, no one has yet produced our substance by

synthetic methods. The complex  will no doubt show a

high degree of physiological activity when it is joined with nitrogenous side chains of widely different chemical structures. An argument as to identity of chemical structures, which rests largely on similarity of physiological action, is of little value when it is remembered that compounds which differ widely in chemical structure not infrequently have one or more physiological reactions in common, such as the power to induce local anæsthesia, or to cause mydriasis.

NOTES ON SOME SYNTHETIC EXPERIMENTS.

Influenced by the point of view which has just been presented, we undertook a series of experiments with the purpose of forming a number of physiologically active compounds which should contain one or two hydroxylated benzene nuclei associated with a nitrogenous complex containing two atoms of nitrogen. Only one of the compounds thus far prepared by us has a pronounced action as a local vaso-constricting agent. We have no

¹ Stolz, *Ber. d. deutsch. chem. Gesellsch.*, xxxvii, p. 4149, 1904. Dakin, *Journ. of Physiol.* (Proc. Physiol. Soc.), February 25, 1905.

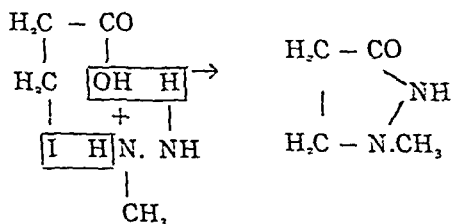
analyses of our products to offer and we give our notes in bare outline in the hope that others may find in them something of value.

METHYLHYDRAZINE ($\text{CH}_3\cdot\text{NH}\cdot\text{NH}_2$) AND β -IODOPROPIONIC ACID.

These substances, the first¹ of which should be thoroughly pure and well dried, are allowed to act on each other in benzene solution. Union at once takes place with the evolution of heat and the formation of water, and the resulting compound falls out as a white viscous substance insoluble in toluene. If the ethyl ester of β -iodopropionic acid is used in place of β -iodopropionic acid an entirely similar product is obtained, but in the course of a few days it shows imbedded in it a large number of colorless prisms.

In either case the substance is very soluble in water and alcohol, insoluble or only slightly soluble in ether and in benzene. The addition of a five per cent. solution of sulphuric acid does not cause β -iodopropionic acid to be liberated. The difficulty we have encountered in freeing these products from hydriodic acid or iodine leads us to surmise that water or alcohol abstraction alone occurred in the above reactions, while the iodine has remained in its position in the iodopropionic acid, and that our substances therefore are open chain hydrazides.

We had reason to hope that the reaction would take place in agreement with the following scheme,



and the product in this case would be 1-methyl 3-pyrazolidone.

These hydrazides, as we shall for the present call them, react easily with protocatechuic aldehyde, vanillin, and chloraceto-

¹ For method of preparation, see G. von Brünig, *Ann. d. Chem.*, ccliii, p 5, 1889.

pyrocatechin. Warmed on the water-bath with vanillin, both hydrazides yield a red crystalline product. Similarly treated with protocatechuic aldehyde, the hydrazide of β -iodopropionic acid yields a non-crystallizable substance which has a slight action as a local vaso-constricting agent, while the hydrazide of the iodopropionic ester is easily obtained in the form of yellow crystals.

In this last case the mixture of aldehyde and hydrazide solidifies to a hard mass when removed from the water-bath. This is softened with water, neutralized with a solution of sodium bicarbonate, whereupon a tar-like precipitate falls out which soon turns into a reddish granular compound. When this compound is treated with a very little dilute hydrochloric acid it is converted into an acid which crystallizes in yellow needles. This acid may be recrystallized from hydrochloric acid. Like the other compound described, it reduces Fehling's solution and ammoniacal solution of silver nitrate and is free of iodine. Its aqueous solutions do not blanch the vessels of the conjunctiva.

ACTION OF THE HYDRAZIDE OBTAINED FROM β -IODOPROPIONIC ACID UPON CHLORACETOPYROCATECHIN¹ ($C_6H_3 \cdot (OH)_2 \cdot CO \cdot CH_2Cl$).

These two substances react upon each other with some vigor when they are intimately mixed by stirring the powdered crystals of the latter compound into the viscous hydrazide. The reacting mixture soon assumes a dark-brown color, a gas is continuously evolved that is found to carry fumes of a halogen acid, but we can not say whether this acid is the only gaseous product evolved. The reaction was also allowed to take place in the presence of a mixture of zinc dust or zinc chloride. After adding sodium acetate, the zinc was removed with hydrogen sulphide, and organic solvents, as alcohol and acetone, were employed to separate the organic product from the inorganic salts, as far as possible. In all these operations the removal of solvents is effected by evaporation under reduced pressure. At one stage in the process the aqueous solution must be repeatedly

¹ For method of preparation, see Dzierzowski, *Chem. Central-Blatt*, 2, 1893, p. 475.

shaken with ether to remove all traces of altered and uncombined chloracetopyrocatechin. The product finally obtained is a brown, viscous, non-crystallizable residue which is acid in character and still contains iodides. This substance, which may not represent a single chemical individual, is nevertheless of interest, as its aqueous solution in the proper concentration induces a prolonged and complete constriction of the conjunctival vessels. One drop of 0.27 per cent. solution applied to the conjunctiva of a dog suffices to blanch the vessels for a short time. Injection of 0.0027 gram (1 c.c. of a 0.27 per cent. solution) into the jugular vein of a dog weighing 7.58 kg. caused the arterial pressure to fall promptly from 150 to 130 mm. An injection of 0.012 gram caused a fall of 90 mm. in the arterial pressure, with a fairly rapid return to the normal pressure. No vagus effect is noticeable in the blood-pressure tracing.

A crystalline and sharply characterized compound which is, however, entirely powerless to cause vaso-constriction when applied locally, is easily obtainable as follows. The hydrazide is first treated on the water-bath, with such a quantity of a ten per cent. solution of nitric acid as is found on trial to be just sufficient to liberate its iodine. At a certain point in the oxidation clouds of iodine vapor are evolved, and if more water is now added and the evaporation continued until the residue is free from nitric acid, it will generally be found to have lost all of its iodine. If this result has been effected without having proceeded too far in the oxidation of the hydrazide, it will be possible to obtain a substance in the form of reddish-brown crystalline crusts by dissolving the residue in hot water, adding a little alcohol, and allowing it to stand. If this substance is boiled in water with chloracetopyrocatechin, an addition product is obtained which crystallizes from the solution in the form of large, reddish, raspberry-like nodules with a diameter of one centimeter or less. On the assumption that the molecular weight of the oxidized hydrazide was not larger than 84, two molecules of chloracetopyrocatechin were used in preparing this compound, and it was found that this quantity had entered into combination without a remainder. The substance thus formed is fairly soluble in water, emits an odor like that of methylamine when treated with alkalies, is neutral to litmus,

and gives no precipitate in the presence of nitric acid on the addition of silver nitrate. It reduces Fehling's solution and ammoniacal solution of silver nitrate. This compound may possibly be of interest to chemists and it will perhaps repay more careful examination. Its action on the arterial pressure has not been tested.

It may also be stated that the iodine of our hydrazide may be removed by treating its solution in 60 per cent. alcohol with an alcoholic solution of cupric acetate and removing the excess of copper with hydrogen sulphide, all evaporations of solvents being effected as before under reduced pressure.

The product thus obtained is a brownish, viscous, non-crystallizable substance which still reduces Fehling's solution but only on prolonged boiling. We have not as yet been able to combine this substance with the hydroxybenzene compounds above named so as to secure well characterized products.

THE INFLUENCE OF THYROID FEEDING UPON POISONING BY ACETONITRILE.

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Speculation as to the functions of the thyroid glands has taken two main directions: some suppose that these organs elaborate an internal secretion which is of itself necessary for certain processes of metabolism, while others think that their chief function is to neutralize poisonous substances originating in the body ("enterotoxines") or introduced into it from without (iodine, for example). In addition to these chemical theories a mechanical function (the prevention of an excessively high blood-pressure in the brain) has been ascribed to these organs. How divergent these various views are is shown by the fact that whereas some writers think that the iodine usually found in the thyroid represents a poisonous substance which has been rendered harmless by its union with proteids, others think these organic iodine compounds are the physiologically important constituents of the organ; in fact it is claimed that the administration of certain organic halogen compounds goes far towards preventing the serious results of the removal of the thyroids.

Notwithstanding the large amount of work which has been done on the thyroids and the many theories as to their functions, there seem to be no experiments on record in which it has been definitely shown that the thyroid can neutralize a poison. Such experiments are recorded in the following pages. Whether or no the poison used in these experiments (acetonitrile), or similar poisons, are involved in any pathological processes (as has been supposed by some), the fact that it is a substance of definite and known chemical composition makes it probable

that an investigation of the manner in which the neutralization occurs will throw light upon the function of the thyroid.

The physiological action of acetonitrile (CH_3CN) has been the subject of a number of investigations. It is believed that its toxicity is due to hydrocyanic acid which is slowly liberated in the organism; part of the hydrocyanic acid appears in the urine as sulphocyanate, while the methyl group undergoes partial oxidation and appears in the urine as formates. One of the most interesting facts in the pharmacology of acetonitrile is that it can be neutralized by various sulphur compounds.¹

Method.—The experiments were performed upon white mice. The thyroid (in the form of the dry powder) was administered in the food by Ehrlich's cake method; the acetonitrile was injected subcutaneously. Control experiments in which the same food without thyroid, or with thyroidectin, peptone, thymus, etc., was given were always made. In another series of experiments thyroid was fed and the animals' susceptibility to hydrocyanic acid or nitroprussiate of soda tested.

A matter of much importance in experiments with acetonitrile is the securing of animals of nearly the same age and weight and which have been kept under the same conditions and upon the same kind of food; I have found that the susceptibility of mice to acetonitrile can be modified greatly by altering these conditions.² In the following experiments these precautions were observed and controls made in each case.

Thyroid and Acetonitrile.—In the first series of experiments 0.3 gramme of the dried and powdered thyroid of the sheep was

¹ For references see *Arch. int. de Pharmodynamie et Therapie*, xii, p. 447, 1904.

² In a series of experiments performed in Professor Ehrlich's laboratory at Frankfort, I found the fatal dose of acetonitrile for mice to be almost uniformly approximately 0.7 mgr. per gramme body weight, although occasionally a lot of mice which had been kept under different conditions, and which were of a different age, were found to be much more susceptible. In Washington I have sometimes obtained mice for which the fatal dose was as small as 0.15 mgr. per gramme body weight. Some of the conditions determining the toxicity of acetonitrile will be discussed in a future communication; the fatal dose for any one lot of mice was remarkably uniform, and could as a rule be definitely determined to within 0.01 to 0.02 mgr. per gramme mouse.

added to each cake of four grammes weight; one of these cakes was given to a mouse each day. The mouse seldom ate an entire cake during a day, and the amount of thyroid eaten was not accurately determined; about 7 per cent. of the food consisted of thyroid. The following experiments (with the controls) may be quoted as examples:

Date.	Weight of Mouse	Remarks.
1905, vi, 7	16.65	Feeding of thyroid cakes (0.3 gm. + 4 gm.) commenced.
14	17.21	
20	17.35	Acetonitrile 24.29 mgr., <i>i. e.</i> , 1.4 mgr. per gm. body weight. Survived.
21		Cakes without thyroid ("plain cakes") fed.
vii, 3	16.82	
4	16.85	Acetonitrile, 11.8 mgr., <i>i. e.</i> , 0.7 mgr. per gm. mouse. Died in 1 hour.
1905, vi, 7	18.42	Thyroid cakes (0.3 gm. + 4 gm.).
14	19.41	
19	18.51	Acetonitrile, 20.36 mgr.; 1.1 mgr. per gm. mouse. Survived.
1905, vi, 7	18.91	Thyroid cakes (0.3 gm. + 4 gm.).
14	14.61	
19	14.55	Acetonitrile, 17.46 mgr.; 1.2 mgr. per gm. mouse. Survived.
1905, vi, 7	21.11	Thyroid cakes (0.3 gm. + 4 gm.).
14	20.25	
19	17.42	Acetonitrile, 24.39 mgr.; 1.4 mgr. per gm. mouse. Died in about 2½ hours.

Thus the fatal dose of acetonitrile for mice whose food had contained about 7 per cent. of thyroid for 12 or 13 days was not less than 1.4 mgr. per gramme mouse.

As control experiments (*i. e.*, experiments in which mice had received the same food without the thyroid) the following may be cited:

Date.	Weight of Mouse.	Remarks.
1905, vi, 7	22.05	Cakes.
14	22.01	
18	22.86	Acetonitrile, 9.14 mgr.; 0.4 mgr. per gm. mouse. Died in 2 to 3 hours.
1905, vi, 7	15.45	Cakes.
14	15.11	
19	14.55	Acetonitrile, 5.08 mgr.; 0.35 mgr. per gm. mouse. Died in 1½ hours.

Date.	Weight of Mouse.	Remarks.
1905, vi, 7	15.62	Cakes.
14	15.41	
19	15.82	Acetonitrile, 5.06 mgr.; 0.32 mgr. per gm. mouse. Died in $2\frac{1}{2}$ hours.
1905, vi, 7	19.62	Cakes.
14	18.81	
18	19.20	Acetonitrile, 5.76 mgr.; 0.3 mgr. per gm. mouse. Survived.

Thus the fatal dose for these mice was about 0.32 mgr. per gramme body weight as contrasted with 1.4 mgr. for mice which had received the thyroid. Similar experiments with similar results will be described later.

Systematic experiments have not been made to determine how long the protective action of thyroid continues. The first experiment in the above series shows that a mouse which, when receiving thyroid, had recovered from 1.4 mgr. of acetonitrile per gm., died from 0.7 mgr. after 14 days during which it received cakes without thyroid.

The following experiment, however, suggests that some degree of protective action continues for at least two weeks.

Date.	Weight of Mouse.	Remarks.
1905, vi, 7	21.02	Thyroid cakes (0.3 gm. + 4 gm.).
14	20.05	
18	20.41	Acetonitrile, 16.33 mgr.; 0.8 mgr. per gm. mouse. Survived.
20	18.82	Plain cakes.
29	17.25	
vii, 3	15.72	Acetonitrile, 9.43 mgr.; 0.6 mgr. per gm. mouse. Survived.

Another experiment which may be quoted in this connection is the following:

Date.	Weight of Mouse.	Remarks
1905, ii, 18	18.65	Thyroid cakes (0.4 gm. + 4 gm.).
27	19.22	
28	17.72	Acetonitrile, 15.95 mgr.; 0.9 mgr. per gm. mouse. Survived.
iii, 1		Thyroid continued.
6	14.82	
10	16.75	Acetonitrile, 15.08 mgr.; 0.9 mgr. per gm. mouse. Survived.
11		Plain cakes.
18	17.35	
24	17.42	Acetonitrile, 10.45 mgr.; 0.6 mgr. per gm. mouse. Died in $3\frac{1}{2}$ hours.

In the second series of experiments the administration of thyroid was continued for but a very short time:

Date.	Weight of Mouse.	Remarks.
1905, vii, 7	14.62	Thyroid cakes (0.3 gm. + 4 gm.).
9	14.54	Acetonitrile, 7.27 mgr.; 0.5 mgr. per gm. mouse. Survived.
1905, vii, 7	11.35	Thyroid cakes (0.3 gm. + 4 gm.).
10	11.45	Acetonitrile, 6.87 mgr.; 0.6 mgr. per gm. mouse. Survived.
1905, vii, 7	20.65	Thyroid cakes (0.3 gm. + 4 gm.).
9	21.59	Acetonitrile, 7.56 mgr.; 0.35 mgr. per gm. mouse. Survived.
1905, vii, 7	11.51	Thyroid cakes (0.3 gm. + 4 gm.).
10	12.55	Acetonitrile, 8.79 mgr.; 0.7 mgr. per gm. mouse. Died in 3 hours.

Control experiments were as follows:

Date.	Weight of Mouse.	Remarks.
1905, vii, 7	15.15	Plain cakes.
9	13.52	Acetonitrile, 6.21 mgr.; 0.4 mgr. per gm. mouse. Died in about 4½ hours.
1905, vii, 7	13.01	Plain cakes.
10	13.41	Acetonitrile, 4.83 mgr.; 0.36 mgr. per gm. mouse. Died in about 2 hours.
1905, vii, 7	12.72	Plain cakes.
10	12.55	Acetonitrile, 3.77 mgr.; 0.3 mgr. per gm. mouse. Died.

These experiments are sufficient to show that the protective action of thyroid is present in two or three days after the beginning of its administration.

In a third series of experiments a much smaller amount of thyroid (0.1 gm. to 4 gm. cakes) was fed; the protective action did not seem to be any less than after larger doses. Two such experiments were as follows:

Date.	Weight of Mouse.	Remarks.
1905, vii, 14	12.31	Thyroid cakes (0.1 gm. + 4 gm.).
20	10.25	
21	9.51	Acetonitrile, 11.41 mgr.; 1.2 mgr. per gm. mouse. Survived.
22		Thyroid cakes continued.
25	9.72	Acetonitrile, 22.36 mgr.; 2.3 mgr. per gm. mouse. Survived.

Date.	Weight of Mouse.	Remarks.
1905, vii, 14	15.21	Thyroid cakes (0.1 gm. + 4 gm.).
20	15.45	Acetonitrile, 9.27 mgr.; 0.6 mgr. per gm. mouse. Survived.
21		Thyroid cakes continued.
24	13.03	Acetonitrile, 20.85 mgr.; 1.6 mgr. per gm. mouse. Survived.

It is not impossible that the high degree of resistance developed in these experiments was due, to a slight extent, to a certain amount of tolerance having been caused by the first dose; but other experiments on the establishment of tolerance did not lend much support to this view.

With a still smaller quantity of thyroid (0.05 gm. to 4 gm. cake) the result was as follows:

Date.	Weight of Mouse.	Remarks.
1905, viii, 18	12.13	Thyroid cakes (0.05 gm. + 4 gm.).
28	8.73	
ix, 1	8.04	Acetonitrile, 8.04 mgr.; 1 mgr. per gm. mouse. Survived.

With cakes alone, the following result was obtained:

Date.	Weight of Mouse.	Remarks.
1905, viii, 18	12.6	Cakes.
28	12.35	
ix, 1	12.5	Acetonitrile, 3 mgr.; 0.24 mgr. per gm. mouse. Died in 2 hours.

Thyroidectin and Acetonitrile.—The theory which led to the introduction into medicine of the blood of thyroidectomized animals (*thyroidectin*), viz., that the blood of such animals contains an excess of a poison normally neutralized by the thyroid, implies that thyroidectin has an action antagonistic to that of the thyroid. Support for this theory has been sought in the favorable reports on the use of thyroidectin in the treatment of exophthalmic goitre, although it should be remembered that the view that the symptoms of exophthalmic goitre are due, in part at least, to excessive thyroid activity is not universally accepted.

A number of experiments were made to determine whether thyroidectin has an action towards acetonitrile the reverse of

that of the thyroid; the experiments offered nothing of special interest and only the main results will be given. In the first series of experiments the thyroidectin was given in the proportion of 0.3 grammes to 4 grammes cake. The results were as follows:

- | | | | | | |
|-----|----------------|----------|--------------|----------|------------|
| (1) | After 11 days, | 0.3 mgr. | acetonitrile | per gm., | fatal. |
| (2) | " 11 " | 0.15 | " | " | " |
| (3) | " 11 " | 0.08 | " | " | not fatal. |

The results after feeding cakes alone were as follows (for details see pp. 35-36, experiments June 18-19):

- | | | | | | |
|-----|----------------|----------|--------------|----------|------------|
| (1) | After 11 days, | 0.4 mgr. | acetonitrile | per gm., | fatal. |
| (2) | " 12 " | 0.35 | " | " | " |
| (3) | " 12 " | 0.32 | " | " | " |
| (4) | " 11 " | 0.3 | " | " | not fatal. |

The fatal dose of acetonitrile for mice which had received thyroid in this series was about 1.4 mgr. per gramme mouse.

In another series of experiments the following results were obtained with thyroidectin (0.3 gm. + 4 gm.).

- | | | | | | |
|-----|---------------|----------|--------------|----------|--------|
| (1) | After 2 days, | 0.4 mgr. | acetonitrile | per gm., | fatal. |
| (2) | " 2 " | 0.35 | " | " | " |
| (3) | " 3 " | 0.2 | " | " | " |

With cakes:

- | | | | | | |
|-----|---------------|----------|--------------|----------|------------|
| (1) | After 2 days, | 0.4 mgr. | acetonitrile | per gm., | fatal. |
| (2) | " 3 " | 0.36 | " | " | " |
| (3) | " 3 " | 0.3 | " | " | " |
| (4) | " 2 " | 0.35 | " | " | not fatal. |
| (5) | " 4 " | 0.25 | " | " | " |

The fatal dose of acetonitrile for mice of this series which had received thyroid was about 0.7 mgr. per gramme mouse.

Although the experiments in the above series are too few to admit of positive conclusions, yet they indicate that thyroidectin has a slight action opposite to that of thyroid, *i. e.*, that it slightly increases the susceptibility of mice to acetonitrile. That this effect is not, however, greater than that of the dried blood of normal sheep¹ or of Witte's peptone is shown by the following experiments. Thus in one series of experiments mice were fed on cakes containing blood, peptone, or thyroidectin in

¹ For the preparation of this blood and for the thyroidectin used in these experiments, we are indebted to the kindness of Parke, Davis & Co.

the proportion of 0.2 gm. to 4 gm. cakes. The following results were obtained:

With blood:

- | | | | | | |
|-----|---------------|----------|--------------|----------|------------|
| (1) | After 6 days, | 0.2 mgr. | acetonitrile | per gm., | fatal. |
| (2) | " 7 " | 0.17 " | " " | " " | not fatal. |
| (3) | " 6 " | 0.15 " | " " | " " | " " |

With peptone:

- | | | | | | |
|-----|---------------|-----------|--------------|----------|------------|
| (1) | After 7 days, | 0.17 mgr. | acetonitrile | per gm., | fatal. |
| (2) | " 7 " | 0.15 " | " " | " " | not fatal. |
| (3) | " 6 " | 0.12 " | " " | " " | " " |

With thyroidectin:

- | | | | | | |
|-----|---------------|----------|--------------|----------|------------|
| (1) | After 7 days, | 0.2 mgr. | acetonitrile | per gm., | not fatal. |
| (2) | " 7 " | 0.17 " | " " | " " | " " |
| (3) | " 7 " | 0.12 " | " " | " " | " " |

With cakes alone:

- | | | | | | |
|-----|---------------|---------|--------------|----------|------------|
| (1) | After 7 days, | 0.3 gm. | acetonitrile | per gm., | fatal. |
| (2) | " 6 " | 0.24 " | " " | " " | not fatal. |

With thyroids:

- | | | | | | |
|-----|---------------|--------|--------------|----------|------------|
| (1) | After 6 days, | 1 mgr. | acetonitrile | per gm., | not fatal. |
|-----|---------------|--------|--------------|----------|------------|

The administration of blood as well as thyroidectin caused, as a rule, a slight loss of weight; this was, however, less marked than after the administration of thyroid.

In another series of experiments (in which young mice, which seem to be more susceptible to acetonitrile than the older ones, were used) the following results were obtained:

With peptone cakes (0.2 gm. + 4 gm.):

- | | | | | | |
|-----|---------------|----------|--------------|----------|--------|
| (1) | After 6 days, | 0.2 mgr. | acetonitrile | per gm., | fatal. |
| (2) | " 6 " | 0.17 " | " " | " " | " " |
| (3) | " 6 " | 0.14 " | " " | " " | " " |

With thyroidectin cakes (0.2 gm. + 4 gm.):

- | | | | | | |
|-----|---------------|----------|--------------|----------|--------|
| (1) | After 6 days, | 0.2 mgr. | acetonitrile | per gm., | fatal. |
| (2) | " 6 " | 0.17 " | " " | " " | " " |
| (3) | " 7 " | 0.14 " | " " | " " | " " |

With blood (0.2 gm. + 4 gm.):

- | | | | | | |
|-----|---------------|-----------|--------------|----------|--------|
| (1) | After 6 days, | 0.25 mgr. | acetonitrile | per gm., | fatal. |
| (2) | " 7 " | 0.21 " | " " | " " | " " |

With thyroid cakes (0.1 gm. + 4 gm.):

- | | | | | | |
|-----|---------------|----------|--------------|----------|------------|
| (1) | After 7 days, | 1.2 mgr. | acetonitrile | per gm., | not fatal. |
|-----|---------------|----------|--------------|----------|------------|

Thyroid in Combination with Thyroidectin, Peptone, etc.—It having been found that thyroid diminishes the susceptibility of mice to acetonitrile, while thyroidectin, peptone, and dried blood tend to increase their susceptibility to this poison, a few experiments were made in which thyroid combined with one of these other substances was fed to mice and then their susceptibility to acetonitrile tested. Three such experiments were as follows:

With thyroid cakes (0.1 gm. + 4 gm.):

- | | | | | | |
|-----|----------------|----------|--------------|----------|------------|
| (1) | After 10 days, | 1.6 mgr. | acetonitrile | per gm., | not fatal. |
| (2) | " 10 " | 2. | " | " " | " " |
| (3) | " 10 " | 2.3 | " | " " | " " |

With cakes containing peptone (0.2 gm.) and thyroid (0.1 gm. to 4 gm.):

- | | | | | | |
|-----|----------------|----------|--------------|----------|------------|
| (1) | After 10 days, | 1.4 mgr. | acetonitrile | per gm., | not fatal. |
| (2) | " 10 " | 1.6 | " | " " | fatal. |

With cakes containing blood (0.2 gm.) and thyroid (0.1 gm. to 4 gm.):

- | | | | | | |
|-----|----------------|----------|--------------|----------|------------|
| (1) | After 10 days, | 1.7 mgr. | acetonitrile | per gm., | not fatal. |
| (2) | " 11 " | 1.9 | " | " " | fatal. |

With cakes containing thyroidectin (0.2 gm.) and thyroids (0.1 gm. to 4 gm.):

- | | | | | | |
|-----|----------------|----------|--------------|----------|------------|
| (1) | After 10 days, | 1.7 mgr. | acetonitrile | per gm., | not fatal. |
| (2) | " 10 " | 1.9 | " | " " | " " |

Thus blood and peptone diminish the action of thyroid in decreasing the susceptibility of mice to acetonitrile; the experiments with thyroidectin were not numerous enough to be conclusive.

Experiments with Thymus, Suprarenals, etc.—A number of experiments were made to determine whether other proteids or preparations of other glands have any effect upon the susceptibility of mice to acetonitrile. Among the substances tried were nutrose, gelatin, casein, nuclein from yeast, the dried thymus and suprarenal glands and the ovaries. Some of these increase the susceptibility to acetonitrile slightly: the suprarenals have an effect as marked as that of peptone; the effects of thymus and nuclein are about equal and distinctly less than that of peptone. The results of the experiments with nutrose and ordinary casein were not closely concordant, but indicate that casein has a more marked effect than has nutrose. Gelatin

and the dried ovaries certainly do not increase the susceptibility of mice to acetonitrile; possibly they decrease it slightly, but this is not entirely clear.

Thyroids, Hydrocyanic Acid, and Nitroprussiate of Soda.—In order to determine whether thyroid has any influence upon the susceptibility of mice towards other cyanogen compounds the following experiments were made with nitroprussiate of soda and hydrocyanic acid:

Date.	Weight of Mouse.	Remarks.
1905, viii, 9	22.87	Thyroid cakes (0.3 gm. + 4 gm.).
15	19.1	Nitroprussiate of soda, 0.344 mgr.; 0.018 mgr. per gm. mouse. Died in 18 minutes.
1905, viii, 9	19.58	Thyroid cakes (0.3 gm. + 4 gm.).
15	15.13	Nitroprussiate of soda, 0.182 mgr.; 0.012 mgr. per gm. mouse. Died in 30 minutes.
1905, viii, 9	17.75	Thyroid cakes, (0.3 gm. + 4 gm.).
15	15.77	Nitroprussiate of soda, 0.126 mgr.; 0.008 mgr. per gm. mouse. Died in about 2½ hours.
1905, viii, 9	16.42	Thyroid cakes, (0.3 gm. + 4 gm.).
15	13.46	
16	13.79	Nitroprussiate of soda, 0.082 mgr.; 0.006 mgr. per gm. mouse. Survived.

The control experiments in which mice received only cakes gave the following results:

- (1) After 6 days, 0.015 mgr. nitroprussiate of soda per gm. mouse, fatal in 30 minutes.
- (2) " 6 " 0.01 " " " " per gm. mouse, fatal in 1 hour 30 min.
- (3) " 7 " 0.007 " " " " per gm. mouse, not fatal.
- (4) " 7 " 0.008 " " " " per gm. mouse, not fatal.

With hydrocyanic acid the results were as follows:

Date.	Weight of Mouse.	Remarks.
1905, vii, 22	21.47	Thyroid cakes, (0.3 gm. + 4 gm.).
29	17.55	Hydrocyanic acid, 0.07 mgr.; 0.004 mgr. per gm. mouse. Died in 3 minutes.
1905, vii, 22	21.28	Thyroid cakes, (0.3 gm. + 4 gm.).
29	19.34	Hydrocyanic acid, 0.0484 mgr.; 0.0025 mgr. per gm. mouse. Died in from 1 to 3 hours.

Date.	Weight of Mouse.	Remarks.
1905, vii, 22	24.79	Thyroid cakes, (0.3 gm. + 4 gm.).
28	19.15	
31	17.41	Hydrocyanic acid, 0.0383 mgr.; 0.0022 mgr. per gm. Survived.
		Thyroid cakes continued.
viii, 1	16.41	Hydrocyanic acid, 0.0443 mgr.; 0.0027 mgr. per gm. mouse. Died.

With plain cakes the results were as follows:

- (1) After 7 days, 0.003 mgr. hydrocyanic acid per gm., fatal in 35 min.
 (2) " 7 " 0.0025 " " " " not fatal.

These experiments show that feeding thyroid did not increase the resistance of mice to nitroprussiate of soda or hydrocyanic acid in the least. On the contrary, there are unmistakable indications that the susceptibility of the animals to these poisons is slightly increased; this is probably due, not to any specific action, but to a lowering of general resistance, for increased susceptibility was observed in other cases after agencies which caused a loss of weight.

Conclusion.—It is not my purpose to discuss at this time the probable cause of the antagonistic action of the thyroid towards acetonitrile; before this can be profitably done further experiments will be necessary. It is possible that the administration of thyroid so alters the metabolism that more sulphur (by which cyanogen compounds are normally neutralized in the body) is made available. The fact, however, that the thyroid has no antagonistic action towards nitroprussiate of soda or free hydrocyanic acid is rather opposed to this supposition, for sulphur compounds which have a neutralizing action towards acetonitrile have, as a rule, a similar action towards nitroprussiate of soda and hydrocyanic acid. This point, as well as a number of others bearing upon this subject, is being investigated at present.¹

One other feature of the effect of thyroid feeding may be mentioned: A number of writers have pointed out an important

¹ The preparation of thyroid used in these experiments was an ordinary commercial one obtained from the sheep; it doubtless contained a certain amount of parathyroid tissue. The latter was probably present in too small quantities to materially affect the results, but this point, as well as the effect of parathyroid feeding, is being investigated.

relationship between the thyroid and proteids. Thyroid-ectomized animals, for example, succumb very quickly when put upon a meat diet. Watson¹ speaks of the thyroids having, microscopically, an "exhausted appearance" when the animals were given a meat diet. In these experiments with acetonitrile a similar antagonistic action was observed; proteids² increase the susceptibility of mice to acetonitrile, while thyroid decreases it.

NOTE DURING PROOF-READING.—Later experiments show that feeding with parathyroids does not decrease the susceptibility of mice to acetonitrile; on the contrary, it causes a distinct increase of their susceptibility to this poison. Whether this increase of susceptibility is greater than that caused by an equivalent amount of proteid has not been definitely determined, but the indications are to this effect. The addition of potassium iodide to the food increases slightly the resistance of mice to acetonitrile; the extent of this action however is not at all comparable to that of thyroid.

¹ *Lancet*, February 11, 1905, p. 347.

² The effect of the different kinds of food upon the resistance of mice to acetonitrile will be discussed in a future communication.

THE CLEAVAGE PRODUCTS OF PROTEOSES.

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Through the work of Kühne, Chittenden, and Neumeister, it was shown that the proteid molecule can be divided into several components, having distinct physical properties. The substances were designated by Chittenden proteoses. Hofmeister and his co-workers have further demonstrated that of the various proteoses the primary pre-exist in the molecule of the native proteid. They have also made it seem probable that the various proteoses possess distinct differences in their composition. These conclusions were based, as a rule, on qualitative color tests. All writers on the subject have also noted that the various proteoses differ in their resistance towards proteolytic enzymes. And Fischer and his co-workers have demonstrated that synthetic polypeptides composed of the lower amido-acids are decomposed by trypsin less readily than polypeptides containing in their molecule the higher acids. The last two observations particularly pointed to the probability that the proteoses obtained on enzymotic cleavage of proteids differ in their composition.

There are, however, comparatively few investigations directed toward the study of the cleavage products of individual proteoses. The oldest and most complete work in this direction is that of Pick,¹ in which he analyzed the comparative composition of proto- and hetero-albumose of Witte's peptone. The conclusions based on this investigation were that the molecule of proto-albumose contains about 25 per cent. of its nitrogen in form of basic substances, that it contains no glyocol, little leucin, and abundant tyrosin. On the other hand, in the molecule of hetero-albumose 39 per cent. of its nitrogen exists in the

¹ Pick, *Zeitschrift f. physiol. Chem.*, xxviii, p. 219, 1899.

form of basic substances; there is little or no tyrosin, but abundant leucin and glycocoll. The presence of aspartic and glutamic acid is doubtful.

Friedmann,¹ Haslam,² and Hart³ analyzed the basic constituents of proteoses and arrived at conclusions similar to those of Pick.

The writer⁴ compared the proportion of glycocoll in primary and secondary gelatoses and in gelatin-peptone, and noted a rise in the proportion in gelatoses.

Thus a systematic and complete analysis of the cleavage products of various proteoses is still lacking.

The present communication contains the results of analyses of proto- and hetero-albumose obtained from Witte's peptone, and, it is hoped, will be followed by analyses of the remaining fractions of Witte's peptone, as well as by that of other proteoses.

PREPARATIONS OF PROTO- AND HETERO-ALBUMOSE.

Several methods of separation of proto- and hetero-albumose were recommended in recent years. The one employed by Pick in Hofmeister's laboratory seemed the most convenient. The process consists in separating the primary albumose from the secondary by means of fractional precipitation with ammonium sulphate, and of a separation of the two primary albumoses based on the difference in their solubility in alcohol. The details recommended by Pick were closely followed by the writer; however, it was noted that the hetero-albumose prepared in this manner is not free from impurities, both mineral and organic. It was therefore considered advisable to subject to further purification the substance obtained by Pick's process. This is best accomplished by dialysis. In this manner a substance is obtained which can be filtered very readily and can be washed on filter paper with distilled water.

The details of preparation of the substances were the following:
A 40 per cent. solution of Witte's peptone was treated with a

¹ Friedmann, *Zeitschrift f. physiol. Chem.*, xxix, p. 51, 1900.

² Haslam, *ibid.*, xxxii, p. 54, 1901.

³ Hart, *ibid.*, xxxiii, p. 347, 1901.

⁴ Levene, *ibid.*, xxxvii, p. 81, 1902.

double volume of 95 per cent. alcohol and allowed to stand overnight. The precipitate thus formed was employed for preparation of the hetero-albumose, the supernatant liquid for that of the proto-albumose. The fraction containing hetero-albumose was dissolved in water and precipitated with an equal volume of a saturated solution of ammonium sulphate. The precipitate was as much as possible freed from adherent liquid, redissolved and reprecipitated four times. The precipitate was finally dissolved in water and treated with a volume of 95 per cent. alcohol, equal to half the volume of the albumose solution. The precipitate thus formed was redissolved and reprecipitated four times. The alcoholic filtrates were added to the proto-albumose fraction. The purified hetero-albumose was dissolved in hot water and dialyzed until it settled as a heavy granular precipitate, which could be filtered very conveniently, and was, unlike other albumose, not very hygroscopic.

Proto-albumose was obtained by following in the main the directions given by Pick. The combined alcoholic filtrates were evaporated to dryness under diminished pressure, dissolved in water, treated with a double volume of alcohol, and allowed to stand overnight. The filtrate was again evaporated to dryness under diminished pressure, dissolved in water, and reprecipitated by means of an equal volume of concentrated ammonium sulphate solution. The operation was repeated four times. The substance was finally dissolved in water, the adherent ammonium sulphate was removed by means of barium hydrate, and the excess of barium by means of carbon dioxide. The solution of proto-albumose was finally concentrated under diminished pressure to a very small volume and precipitated with an excess of 95 per cent. alcohol, and the precipitate repeatedly extracted with alcohol.

About twenty-five pounds of Witte's peptone were employed for the preparation of the albumoses.

HYDROLYSIS OF PROTO-ALBUMOSE.

The alcohol-wet substance was employed for hydrolyzing. It was dissolved in 1800 c.c. of strong hydrochloric acid. Three

c.c. of the solution were employed for a Kjeldahl nitrogen estimation. It required 51.7 c.c. $\frac{N}{10}$ H_2SO_4 for neutralization. Thus there were used for the experiment 278.0 grams of the dry proto-albumose. The decomposition was performed in the usual manner. The product of hydrolysis was esterified according to Fischer's directions. In order to separate the amido-acids from the other substances, an attempt was made to employ the method recently recommended by Skraup.¹ According to this method, the hydrochloric salts of the ethylesters of amido-acids are removed by means of a mixture of alcohol and ether. The residue thus formed contains, according to Skraup, not only hexon bases, but also a number of other substances, discovered by that investigator. The extract containing the amido-acids was concentrated under diminished pressure, and the free esters obtained by the process described by Fischer. It should be remarked here that Skraup's method of separating amido-acids from the other substances did not appear very satisfactory. Again, the method of separating the amido-acids by means of phosphotungstic acid is very imperfect, particularly when the quantity of material available for the experiment is small. For the foregoing condition it seemed most desirable to find a way by which the residue remaining after extraction of the free amido-acids by means of ether could be conveniently employed for further work.

In Fischer's process the hydrochloric esters are transformed into the free by means of sodium hydrate and potassium carbonate. This leaves a residue containing potassium and sodium in excess of the organic substances, and thus renders the analysis of the organic part of the residue practically impossible. It was therefore thought advantageous to substitute the use of potassium carbonate and sodium hydrate by that of a concentrated solution of barium hydrate and of powdered dry barium oxide. An experiment on gelatin with this modification gave very satisfactory results. Thus 650 grams of gelatin yielded 212 grams of free esters distilled at 18 mm. pressure, and 240 grams were obtained from 1 kilo of the proteid in Fischer's laboratory by the original process. However, this modification

¹ Skraup, *Zeitschrift f. physiol. Chem.*, xlix, p. 274, 1904.

was introduced after the present investigation was practically completed.

Mention is made of these circumstances for the reason that the extraction of the esters by Skraup's method, as well as the comparatively high pressure under which the distillation of the esters was performed, has undoubtedly lowered the yield of the esters, and thus the figures obtained in this investigation can be regarded as the minimal figures obtainable, and do not represent the actual proportions present in the proteid molecule.

The esters were distilled at 15 mm. pressure and the following fractions were obtained:

45°—60° C.	—	5.	gr.
60°—80°	—	8.5	"
80°—95°	—	8.3	"
95°—110°	—	7.0	"
110°—135°	—	17.3	"
135°—180°	—	17.2	"
		<hr/>	
Total . . .		63.3	gr.

FRACTION 45°–60° C.

This fraction contained glycocoll, alanin, and perhaps higher amido-acids. It was saponified by boiling with water, evaporated to dryness, and the dry residue again esterified in the usual manner. The hydrochloric salt of glycocoll crystallized. Once recrystallized out of alcohol, it had the melting-point of 144° C. The yield was 1 gram.

The filtrate from the glycocoll ester was again saponified, the hydrochloric acid removed by silver sulphate, and the sulphuric acid by means of barium. The remaining solution was evaporated to dryness. Out of the residue, by repeated fractional distillation, a substance with the composition of alanin was obtained.

0.142 gr. of the substance dried in xylol bath gave on combustion 0.2080 gr. of CO₂ and 0.0993 gr. of H₂O.

For

C₃H₇NO₂—

Calculated: C=40.45 per cent. H=7.87 per cent.

Found: C=39.97 " " H=7.71 " "

FRACTIONS 60°-80° AND 80°-95° C.

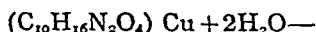
contained leucin, perhaps the lower amido-acids, and prolin.

The two fractions were saponified in the usual manner, evaporated to dryness, and extracted with alcohol. The alcoholic extract was evaporated to dryness and the extraction once repeated. The second extract was again evaporated to dryness. In order to identify prolin it was considered advisable to transform it into the inactive modification. The residue (2 grams) was taken up for that purpose with 3 grams of barium hydrate and 25 c.c. of water, and heated in an autoclave at 140° C. for five hours. The barium was then removed by means of sulphuric acid, concentrated to dryness, the residue extracted with hot absolute alcohol, and the extract again concentrated to dryness. This residue was employed for the preparation of the copper salt of prolin. Once recrystallized from water it possessed all the properties of the copper salt of the inactive acid.

0.1363 gr. of the substance lost on drying in xylol bath 0.0150 gr. in weight.

0.1285 gr. of the substance gave 0.0320 gr. of CuO.

For



Calculated: $\text{H}_2\text{O} = 10.99$ per cent. $\text{Cu} = 19.27$ per cent.

Found: $\text{H}_2\text{O} = 11.00$ " " $\text{Cu} = 18.84$ " "

The remaining part of fraction 60°-80° C. was employed for the preparation of the copper salt of amido-butyric acid. The substance obtained in this manner was very impure. The corresponding part of fraction 80°-95° C. was employed for the preparation of leucin. The substance was obtained in pure condition in the usual manner. The yield was 8 grams.

0.1306 gr. of the substance gave on combustion 0.2625 gr. of CO_2 and 0.1200 of H_2O .

For



Calculated: $\text{C} = 54.96$ per cent. $\text{H} = 9.92$ per cent.

Found: $\text{C} = 54.81$ " " $\text{H} = 10.20$ " "

FRACTION 95°-110° C.

contained chiefly leucin.

FRACTIONS 110°-130° C. AND 130°-180° C.

were employed for preparation of phenylalanin, aspartic and glutamic acids. Both fractions were treated in the same manner; the esters were dissolved in ether and repeatedly shaken with distilled water. Phenylalanin remained in ethereal solution and the other acids were extracted with water.

The saponification of phenylalanin was accomplished by means of hydrochloric acid. The free substance was employed for identification. The yield was about 1 gram.

0.1366 gr. of the substance gave on combustion 0.3255 gr. of CO_2 and 0.0794 gr. of H_2O .

For



Calculated: C=65.45 per cent. H=6.66 per cent.

Found: C=64.98 " " H=6.46 " "

The aqueous extracts of the two fractions were saponified by means of barium. Aspartic acid was obtained in the form of the barium salt of the inactive acid and identified as the free acid.

0.1980 gr. of the substance gave on combustion 0.2587 gr. of CO_2 and 0.0944 gr. of H_2O .

For



Calculated: C=36.09 per cent. H=5.26 per cent.

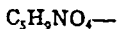
Found: C=35.63 " " H=5.29 " "

There were obtained 2.2 grams of aspartic acid.

Glutamic acid was obtained as the hydrochloride and identified as the free acid.

0.1544 gr. of the substance gave on combustion 0.2300 gr. of CO_2 and 0.0834 gr. of H_2O .

For



Calculated: C=40.81 per cent. H=6.12 per cent.

Found: C=40.63 " " H=6.00 " "

The yield of the hydrochloride was 4.0 grams.

The residue remaining after extraction of the amido-acids, according to Skraup's process was taken up in hot water, acidulated with sulphuric acid, and treated with a 50 per cent. solution of phosphotungstic acid. An oily sediment formed on addition of the reagent. After some time the character of the precipitate forming on addition changed, acquiring the character of a granular powder. The treatment with phosphotungstic acid was discontinued, the supernatant liquid decanted from the oily precipitate, and the treatment with phosphotungstic acid continued until further addition of the reagent no longer caused the formation of a precipitate. The first precipitate on cooling solidified into a hard, tenacious mass. It was not possible to accomplish its solution in ammonia water; in hot baryta water also the solution was incomplete.

The soluble part was freed from barium in the usual manner, concentrated, and treated with silver nitrate solution and baryta water according to the method for the separation of hexon bases devised by Kossel and Kutscher. However, it was impossible to obtain histidin from the corresponding fraction, nor was it possible to obtain arginin from the second silver fraction. The corresponding fraction was repeatedly extracted with boiling alcohol until a dry, brittle residue was formed; but no crystalline compound of the substance was obtained. An aqueous solution of the substance heated with copper oxide formed a blue liquid, which after evaporation to dryness was extracted with alcohol, dried, and analyzed. Its composition was the following:

0.1952 gr. of the substance gave on combustion 0.1952 gr. of CO_2 and 0.0652 gr. of H_2O .

0.2019 gr. of the substance was employed for a Kjeldahl nitrogen estimation. It required for neutralization 23.3 c.c. of sulphuric acid (1 c.c. = 0.001418 N).

0.1952 gr. of the substance gave 0.0255 CuO .

The copper salt was then freed from its copper in the usual manner, the solution concentrated to a very small volume, and treated with a concentrated solution of silver nitrate and absolute alcohol. The solution remained clear. On the addition of a few drops of ammonia an amorphous precipitate formed. It

was washed and dried in the usual manner and had the following composition:

0.1257 gr. of the substance contained 0.0516 gr. of Ag.

0.1580 gr. of the substance was employed for a Kjeldahl nitrogen estimation. It required for neutralization 11.5 c.c. of sulphuric acid (1 c.c. = 0.001418 N).

The composition of the substance corresponded best to the empirical formula $C_{12}H_{22}N_4O_5$.

For



Calculated: C = 39.67 per cent. H = 5.51 per cent. N = 15.45 per cent.
Cu = 17.35 per cent.

Found: C = 40.14 per cent. H = 5.39 per cent. N = 16.40 per cent.
Cu = 15.30 per cent.

For



Calculated: N = 10.85 per cent. Ag = 41.85 per cent.

Found: N = 10.32 " " Ag = 41.11 " "

However, under the conditions of preparation of the substance it is difficult to form an opinion as to its purity or composition.

The second phosphotungstic precipitate was treated in the usual manner for separation of the hexon bases. Again histidin could not be isolated, but arginin and lysin were obtained without difficulty. However, for quantitative estimation of the bases it was considered advisable to employ a separate lot of the albumose. The alcohol-wet substance was employed for hydrolysis. It was dissolved in 525 c.c. of strong hydrochloric acid; 3 c.c. of the solution were employed for a Kjeldahl nitrogen estimation. It required for neutralization 55.8 c.c. of sulphuric acid (1 c.c. = 0.00124 N). Thus 79.66 grams of the albumose were employed for the experiment. The decomposition was performed in the usual manner, excess of acid was then removed by means of lead oxide, and the lead removed by hydrogen sulphide, and from the concentrated solution tyrosin was allowed to crystallize. One gram of the pure substance was obtained. It had the following composition:

0.1147 gr. of the substance gave on combustion 0.2490 gr. of CO_2 and 0.0611 gr. of H_2O .

For



Calculated: C = 59.66 per cent. H = 6.07 per cent.

Found: C = 59.29 " " H = 5.91 " "

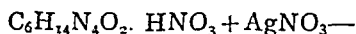
The filtrate from the tyrosin was treated for hexon bases in the usual manner with phosphotungstic acid, and further with silver nitrate and baryta water according to the method of Kossel and Kutscher.

The histidin fraction contained 0.372 grams of nitrogen, or 3.1 per cent. of the total nitrogen. However, it was impossible to obtain pure histidin, although the purification by Hopkins' reagent was applied. The fraction was then reprecipitated by phosphotungstic acid, and again treated in the usual manner, but the attempt to obtain the hydrochloride of histidin was not successful.

The arginin fraction contained 1.16 grams of nitrogen of 9.66 per cent. of the total nitrogen. The carbonate, nitrate, and silver-nitrate salts of the substance were readily obtained from this fraction. One-half of the fraction yielded 3.7 grams of the silver salt.

For identification the silver salt was employed. 0.1345 gr. of the substance gave 0.0355 gr. of Ag.

For



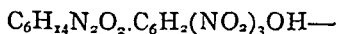
Calculated: Ag = 26.54 per cent.

Found: Ag = 26.40 " "

From the lysin fraction 1.8 grams of the picrate of the base were obtained.

0.2069 gr. of the substance gave on combustion 0.2910 gr. of CO_2 and 0.0929 gr. of H_2O .

For



Calculated: C = 38.40 per cent. H = 4.53 per cent.

Found: C = 38.35 " " H = 4.99 " "

Thus the results of the foregoing analysis are in harmony with the conclusions of Pick, of Friedman, and of Hart regarding the composition of the basic constituents of the proto-albumose; however, they do not harmonize with the conclusions of Pick that the substance contains no glycocoll, little leucin, and abundant tyrosin.

HYDROLYSIS OF HETERO-ALBUMOSE.

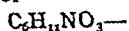
The air-dry substance was employed for the experiment. It was dissolved in 1100 c.c. of concentrated hydrochloric acid.

Two c.c. were employed for a Kjeldahl nitrogen estimation; it required for neutralization 40.0 c.c. of sulphuric acid (1 c.c. = 0.00124 N). Thus, 157.68 grams of the substance were employed for cleavage. The hydrolysis was performed in the usual manner and the hydrochloric acid removed by means of lead oxide as in the experiment with proto-albumose. On concentration tyrosin crystallized. Two grams of the pure substance were obtained. It should, however, be remarked that not all the tyrosin was removed in this manner from the solution.

The recrystallized substance had the melting-point of 315° C.

0.1119 gr. of the substance was employed for a Kjeldahl nitrogen estimation. It required for neutralization 7.25 c.c. of sulphuric acid (1 c.c. = 0.00124 N).

For



Calculated: N = 7.73 per cent.

Found: N = 8.03 " "

The filtrate from tyrosin was acidulated with sulphuric acid and treated with a 50 per cent. solution of phosphotungstic acid as long as a precipitate formed. From the filtrate phosphotungstic acid was removed in the usual manner; the solution was then concentrated and esterified by Fischer's method. The free esters were distilled at 18 mm. pressure. The following fractions were obtained:

50° — 70° C. —	2.5 gr.
70° — 80° C. —	1.5 "
80° — 95° C. —	4.3 "
95° — 105° C. —	2.5 "
105° — 145° C. —	8.7 "
145° — 190° C. —	6.6 "

Total . . . 26.1 gr.

Apparently the treatment with phosphotungstic acid and the operations required for removal of the reagent were combined with considerable loss of material, since the yield of esters was so surprisingly small. The esters were saponified and treated in exactly the same manner as for the analysis of proto-albumose; hence the details will be omitted.

FRACTION 50°-70° C.

contained glycocoll and alanin. Two hundred thousandths of a gram of the glycocoll-ethylester hydrochloride was obtained with m.p. = 144 °C. From the filtrate a substance with the properties of alanin could be isolated.

0.1426 gr. of the substance was employed for a Kjeldahl nitrogen estimation. It required for neutralization 18.1 c.c. of sulphuric acid (1 c.c. = 0.00124 N).

For



Calculated: N = 15.73 per cent.

Found: N = 15.92 " "

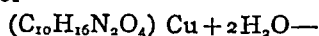
FRACTIONS 70°-105° C.

were all combined, saponified, evaporated to dryness, and extracted with absolute alcohol. The alcoholic extract was treated in the same manner as the corresponding extract of proto-albumose.

A copper salt with the properties of inactive prolin was obtained.

0.098 gr. of the air-dry substance on drying in xylol bath lost in weight 0.011 gr.

For



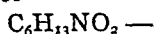
Calculated: H_2O = 10.99 per cent.

Found: H_2O = 11.22 " "

The alcoholic residue was employed for obtaining leucin. Three grams of the substance were obtained.

0.1530 gr. of the substance gave on combustion 0.3070 gr. of CO_2 and 0.1387 gr. of H_2O .

For



Calculated: C = 54.96 per cent. H = 9.92 per cent.

Found: C = 54.70 " " H = 10.00 " "

FRACTION 105°-145° C.

was employed for the obtaining of aspartic and glutamic acids. The phenylalanin fraction on treatment with hydro-

chloric acid gave a very small residue and it was not attempted to prepare the free acid.

Aspartic acid. Only 0.4 gram of the free inactive acid were obtained.

0.1365 gr. of the substance gave on combustion 0.1824 gr. of CO_2 and 0.685 gr. of H_2O .

For

$\text{C}_4\text{H}_7\text{NO}_4$ —

Calculated: C=36.09 per cent. H=5.26 per cent.

Found: C=36.44 " " H=5.57 " "

FRACTION 145° – 190° C.

This fraction was combined with the preceding fraction and served for preparation of aspartic and glutamic acids.

Glutamic acid was obtained as the hydrochloride and identified as the free acid. Two and eight-tenth grams of vacuum-dry substance was obtained.

0.1747 gr. of the substance gave on combustion 0.2620 gr. of CO_2 and 0.0918 gr. of H_2O .

For

C_5HNO_4 —

Calculated: C=40.81 per cent. H=6.12 per cent.

Found: C=40.84 " " H=5.84 " "

For the analysis of the basic constituents the phosphotungstic precipitate was employed. It was decomposed in the usual manner and contained 10 grams of nitrogen, or 38.05 per cent. of the total nitrogen.

The *histidin* fraction contained 1.06 grams of nitrogen, or 4 per cent. of the total nitrogen. The attempt to crystallize the hydrochloride of the base was not successful.

The *arginin* fraction contained 2.8 grams of nitrogen, or 10.60 per cent. of the total.

The arginin was identified as the silver salt.

0.1612 gr. of the substance gave 0.0430 gr. of Ag.

For

$\text{C}_6\text{H}_{14}\text{N}_4\text{O}_2 \cdot \text{HNO}_3 + \text{AgNO}_3$

Calculated: Ag=26.54

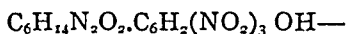
Found: Ag=26.65

It should be remarked, however, that the arginin salts did not crystallize as readily from the fraction as they did from the corresponding one of the proto-albumose.

Lysin was obtained in form of the picrate. The yield was 12 grams.

0.1270 gr. of the substance gave on combustion 0.1790 gr. of CO_2 and 0.055 gr. of H_2O .

For



Calculated: C = 38.40 per cent. H = 4.53 per cent.

Found: C = 38.44 " " H = 4.81 " "

Thus again the foregoing results of analysis of hetero-albumose are in harmony with the results obtained by other observers, but they fail to corroborate the statement of Pick that hetero-albumose, in distinction from the proto-, contains abundant glycocoll and leucin, and very little or no tyrosin. According to Pick, hetero-albumose resembles in its composition gelatin, which does not accord with the present work.

There is also some difference between the results obtained by Hart and those of the foregoing analysis. According to Hart, proto- and hetero-albumose differ principally in the proportion of arginin in their molecule. In the present analysis the most striking difference was noted in the proportions of lysin.

This may be partly explained by the fact that Hart did not separate the individual bases, but estimated the nitrogen in the various fractions.

IS PROTAGON A MECHANICAL MIXTURE OF SUBSTANCES OR A DEFINITE CHEMICAL COMPOUND ?¹

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¹ An abstract of a preliminary report of this investigation was pub-
lished in the *Proceedings of the American Physiological Society*, December,
1904: *American Journal of Physiology*, 1905, xiii, p. xxxv.

I.—GENERAL HISTORICAL REVIEW.

Forty years have passed since Liebreich (1865),¹ working in Hoppe-Seyler's laboratory, separated from brain the material he called protagon. On decomposition with boiling baryta water, protagon was found to yield, among other products, glycerophosphoric acid and fatty acid, substances that Gobley (1847-50), about twenty years earlier, had obtained from "viscous matter," the immediate precursor of lecithin.

Liebreich's observations led immediately to universal acceptance of the conclusion that lecithin, from whatever source it had previously been obtained, was merely "impure protagon."² Hoppe-Seyler was one of the first to endorse this view. In one of the earliest of his papers on the chemistry of blood, which was published shortly after the announcement of Liebreich's results, Hoppe-Seyler³ expressed the opinion that proteids, cholesterin, and protagon were substances of universal distribution in organisms. He also stated his belief that these three substances were intimately connected with the most important processes occurring in *all* animal and vegetable cells.

Shortly after expressing these views, however, Hoppe-Seyler appeared to doubt their validity so far as they related

¹ See the bibliography at the end of this paper.

² "Obgleich ich über das Vorkommen des Protagon im thierischen und pflanzlichen Organismus keine Untersuchungen angestellt habe, so lässt sich doch mit Sicherheit schliessen, dass es ein im Organismus überhaupt verbreiteter Körper ist. Ueberall dort, wo von früheren Autoren Glycerinphosphorsäure, Oleophosphorsäure, Cerebrin u. s. w., überhaupt die sogenannten 'räthselhaften phosphorhaltigen Fette' erwähnt sind, scheint das Protagon im Spiele zu sein, also im Dotter der Eier, Eiter, Sperma u. s. w."—Liebreich, p. 39. See also Diaconow, *Hoppe-Seyler's medicinisch-chemische Untersuchungen*, 1866-71, p. 222. During the short period referred to above, the amount of protagon in any medium was commonly calculated from the quantity of phosphorus contained in the ether extract or ether-alcohol extract. See, in the same volume, Hoppe-Seyler, pp. 146 and 162; Parke, p. 175; and Talmatscheff, p. 276. Also Fischer, *Centralblatt für die medicinischen Wissenschaften*, 1865, p. 225; 1868, p. 659; Hermann, *Archiv für Anatomie und Physiologie*, 1866, p. 27; Kühne, *Lehrbuch der physiologischen Chemie*, 1868, p. 193; footnote (this paper), No. 4, p. 61.

³ Hoppe-Seyler, *Medicinisch-chemische Untersuchungen*, 1866, pp. 141 and 149.

to protagon.¹ This feeling of uncertainty was obviously increased by Parke's² observation that egg-yolk contained, apparently, two different phosphorized organic substances, neither of which was glycerophosphoric acid and at least one of which was unlike protagon. The weight of protagon in Parke's alcoholic extracts, if calculated from the weight of the phosphorus found in them, would have amounted to more than the total quantity of solid matter in the extracts. In a paper following Parke's, Hoppe-Seyler³ stated that egg-yolk contained lecithin but was entirely free from protagon. He also announced that a new investigation by him of the organic phosphorized constituent present in the ether-extract of red corpuscles showed conclusively that protagon was entirely absent from such an extract of the corpuscles, but that, instead, lecithin or a closely related body was contained in it. Although Hoppe-Seyler did not deny the possibility that protagon might be present in the *living* corpuscles, as he had previously believed, and that lecithin might be formed from such protagon by the ether employed in the extraction process, nevertheless he emphasized the fact that there was very little if any reason for assuming that an indifferent reagent like ether could bring about decomposition of a product like protagon. Several additional papers⁴ from the Tübingen laboratory shortly afterward announced results that were in harmony with the tendency to discredit much that had been concluded regarding protagon, although Hoppe-Seyler⁵ still appeared to consider protagon a constituent of the brain.

About a year after the announcement of the data referred to above, however, Diaconow (1868), working under Hoppe-Seyler's supervision, brought forward evidence indicating that

¹ Hoppe-Seyler, *loc. cit.*, p. 176.

² Parke, *Hoppe-Seyler's medicinisch-chemische Untersuchungen*, 1867, p. 213.

³ Hoppe-Seyler, *loc. cit.*, 1867, p. 218.

⁴ Diaconow, *Hoppe-Seyler's medicinisch-chemische Untersuchungen*, 1867, p. 277; also, *Centralblatt für die medicinischen Wissenschaften*, 1867, p. 673. In the first of these papers Diaconow showed very clearly that neither the presence nor the quantity of protagon in a mixture could be ascertained by determinations of the content of phosphorus—a result in accord with Parke's.

⁵ Hoppe-Seyler, *loc. cit.*, p. 220.

brain protagon was merely "impure *lecithin*," or equivalent to a mechanical mixture consisting of Goble's lecithin and phosphorus-free fatty matter similar to if not identical with Müller's (1858) cerebrin.¹ Liebreich had concluded from his discovery of protagon, that lecithin, glycerophosphoric acid, etc., and also substances of the cerebrin type obtainable from brain, did not occur *preformed* in the brain, but were decomposition products of pre-existent protagon. Diaconow's results, on the contrary, seemed to show that Liebreich's conclusion, so far at least as it related to lecithin, was unfounded.

The two investigations on which rested this noteworthy disagreement as to the characters of brain protagon were carried out, as has already been indicated, in Hoppe-Seyler's laboratory and under Hoppe-Seyler's supervision. Hoppe-Seyler himself, and all physiological chemists after him, immediately took Diaconow's view of the nature of protagon, whether from brain or any other source.²

Hoppe-Seyler and his pupils ignored practically all of the work done by previous investigators of the phosphorized fats of the brain. Few writers since have seen fit to call attention to any of the significant data in this connection, except Frémy's (1841), that were obtained prior to Liebreich's preparation of protagon. It is evident to us, after a recent detailed study of the entire literature of the subject, that Fourcroy's (1793) "yellowish-white matter" and Vauquelin's (1813) "white matter" were practically identical and consisted chiefly of cholesterin, other ether-soluble products, and protagon. It is also clear that Gmelin's³ pulverulent brain wax, Kühn's⁴

¹ See also Diaconow, *Centralblatt für die medicinischen Wissenschaften*, 1868, vi, p. 794; also Strecker, *ibid.*

² See further contributions from the Tübingen laboratory in Hoppe-Seyler's *medicinisch-chemische Untersuchungen* by Jüdel, 1869, p. 387; Hoppe-Seyler, 1869, p. 392; Diaconow, 1869, p. 405; Miescher, 1870, p. 450; Hoppe-Seyler, 1870, p. 487; Miescher, 1870, p. 503; Plósz, 1870, p. 522; Hoppe-Seyler, 1870, p. 552 *et seq.*; also, p. 566 *et seq.* Also Petrowsky, *Archiv für die gesammte Physiologie*, 1873, vii, p. 367. See footnote No. 2, p. 60.

³ We have not had access to Gmelin's original paper, but rely upon the abstract of it given by Thudichum (1874).

⁴ The preceding footnote applies to our knowledge of Kühn's product. See also Berzelius, *Lehrbuch der Chemie* (translated by Wöhler), 1840, ix, p. 174.

myelokon, Couerbe's (1834) *cérébrote*, Frémy's (1841) *cerebric acid*, and Goble's (1850) *cerebrin* were practically the same as protagon, *i. e.*, mechanical mixtures in different proportions of about the same phosphorized and non-phosphorized fatty products. In the following summary are given the figures for elementary composition of such of the above-named products as were subjected to analysis; also the figures representing the composition of the original protagon and a recently prepared protagon:

	C	H	N	P	S
Couerbe's <i>cérébrote</i> (1834)...	67.82	11.10	3.40	2.33	2.14 ¹
Frémy's <i>cerebric acid</i> (1841)...	66.70	10.60	2.40	0.90	—
Goble's <i>cerebrin</i> (1850)...	66.85	10.82	2.29	0.43	—
Liebreich's protagon (1865)...	66.74	11.74	2.80	1.23	—
Cramer's protagon (1904)....	66.35	10.98	2.29	1.04	0.71

Of the above-named products *cérébrote* was evidently as much like Liebreich's protagon as any protagon made by a subsequent author, for, although Couerbe obtained *cérébrote* by two general methods, one of them was practically the same as Liebreich's.² In 1865 it must have been evident that Couerbe's imperfect analytic methods might have accounted for the lack of agreement between the data for elementary composition of *cérébrote* and protagon. Couerbe's results were not even mentioned by Liebreich, however,³ and another unnecessary addition was made to chemical nomenclature. The agreement between the figures for elementary composition of *cerebric acid*, *cerebrin* (which Goble regarded the same as *cerebric acid*), and protagon is very striking. But in spite of that and other resemblances to protagon, *cerebric acid* must have been a mixture of somewhat

¹ Couerbe's results were obtained at a time when analytic methods were very imperfect. Frémy attributed the presence of sulfur entirely to proteid impurity, but such could not have been the case, even if half the *cérébrote* product had been proteid. The method of preparation made the presence of albuminous matter quite improbable. It is more reasonable to suppose that reagents containing sulfate were used in the analysis. That sulfur was contained in all the products named in the above summary is now almost certain.

² Extraction was made with *boiling* alcohol, which, it was soon shown, does not readily decompose protagon: Gamgee (1880); also p. 78.

³ Liebreich dismissed all previous products similar to protagon as "decomposition products." This was clearly a mistake.

special proportions, perhaps containing a number of different (isomeric ?) constituents, or possibly modified ingredients, for it was purified by *solution in boiling ether* and recrystallization therefrom. Boiling ether has since been shown to dissolve some of the constituents of protagon but to be without solvent action on the others.

Thudichum appears to have been the only investigator of neurochemical problems who made a thorough study of the work of his predecessors and profited by it. Quite independently his results led him to the same general conclusion as that arrived at by Diaconow, *i. e.*, that protagon was a mechanical mixture. Instead, however, of considering, as did Diaconow, that protagon consisted of lecithin and cerebrin, Thudichum (1874) wrote as follows: "Protagon was a mixture of much cerebrin, phrenosin, kersin, etc., with myelin and some kephalin, also some cholesterol. It was, therefore, mainly Couerbe's *cérébrote*, Frémy's cerebrie acid,¹ but contained a little more myelin than these preparations, which raised its phosphorus a little higher" (page 209).

The above conclusions were expressed by Thudichum in 1874. Bourgoin (1874) also regarded protagon as a mixture. The agreement of Thudichum and Bourgoin with the fundamental point in the observations of Hoppe-Seyler and his pupils (after Diaconow's work) seemed to bury protagon beyond resurrection. But the "protagon question" was soon suddenly opened again by Gamgee and Blankenhorn (1879), who announced a complete confirmation of Liebreich's results and presented data indicating that Diaconow's main conclusions had been erected on faulty observations. Notwithstanding Thudichum's very strenuous and frequently expressed insistence, based as it was upon his own elaborate researches year after year, that protagon was a mixture, physiological chemists in a body followed Gamgee and Blankenhorn to the original position held by Liebreich, and accepted without question, apparently, the view that had been just as readily and generally² discarded previously, *viz.*, that protagon was a definite chemical substance and a leading constituent of the brain.

¹ Thudichum demonstrated later that *cérébrote* and cerebrie acid were not the same.

For about two decades after the publication of the results obtained by Gamgee and Blankenhorn, the conclusions arrived at by these observers were confirmed in the main by the investigations of Geoghegan (1879), Parcus (1881), Baumstark (1885), Kossel and Freytag (1893), Ruppel (1895), Gulewitsch,¹ Zuelzer (1899), Noll (1899), Ulpiani and Lelli (1902). Nobody seemed to avow agreement with Thudichum.² During the same period, until his death a few years ago, Thudichum stood practically alone in maintaining the correctness of the conclusion that protagon was a mechanical mixture, and he frequently assailed, with increasing bitterness, most of the above-named investigators for their failure to agree with him (1879-1901). Thudichum's unsparing denunciation of those of his colleagues who opposed his views and his indiscriminate disparagement of their labors appear to have been among the causes which prevented, until recently, the attention and appreciation that very much of his own elaborate work undoubtedly deserves. In our opinion, Thudichum has made more important contributions to neurochemistry than any other investigator.

In 1899 Hammarsten³ indicated in the following language the feeling then prevalent among physiological chemists regarding Thudichum's work: "Thudichum claims to have isolated from the brain a number of phosphorus-containing substances which he divides into three main groups: kephalins, myelins, and lecithins. Thus far his results have not been confirmed by any other investigators."⁴ Recently, however, many of Thudichum's results have been confirmed by Wörner and Thierfelder (1900), Koch (1902), Lesem and Gies (1902), Bethe,⁵ and others. Our own work confirms some of them.

¹ Cited by Noll (1899).

² Some of the results obtained by Gamgee, by Parcus, and by Kossel and Freytag were confirmations of Thudichum's dissenting conclusions, although they have not been regarded as such.

³ Hammarsten, *Lehrbuch der physiologischen Chemie*, 1899, p. 366. In the last edition of the same work (1904) Hammarsten says that Thudichum's investigations are without doubt of great importance.

⁴ Some of the results, as we have already indicated, were confirmations, although they have not been so regarded.

⁵ Bethe, *Archiv für experimentelle Pathologie und Pharmakologie*, 1902, xlviii, p. 73.

Five years ago the work of Wörner and Thierfelder seemed to afford ample verification of Thudichum's claims and to show beyond a doubt that protagon was an indefinite mechanical mixture as he had so long contended. The subsequent investigation by Lesem and Gies, which had been in progress before the publication of Wörner and Thierfelder's research, gave further emphasis to this conclusion. In his recent extensive study of the quantitative composition of the brain, Koch¹ remarked: "Protagon, I will not attempt to resurrect, as the work of Thierfelder and of Gies has settled its fate." In the last edition of his text-book, Hammarsten² states that the investigations of the same observers "show that protagon is not an individual substance but a mechanical mixture."

This brief review, to the year 1904, of the general facts bearing on the individuality of protagon reveals a singular history. Liebreich's protagon was hailed, in 1865, and during the next few years, as a new and remarkable substance, from which such compounds as lecithin, wherever they occurred, were formed by decomposition, when in reality it was practically the same as several products previously obtained from brain (page 63). For a decade after Diaconow's work in 1868, protagon was universally regarded as a mechanical mixture—merely "impure lecithin," as one investigator put it. In 1879 protagon was resurrected by Gamgee and Blankenhorn, and again declared to be a definite chemical substance of fundamental physiological importance. For twenty years afterward the work and polemics of Thudichum failed to shake the universal confidence in the chemical integrity of protagon, although Gamgee obtained pseudocerebrin from it and Kossel and Freytag concluded that there were several protagons. In 1900 protagon was once more seemingly shown by Wörner and Thierfelder to be a variable product, and during the ensuing five years it was again generally regarded as merely a mechanical mixture.

Last year the protagon question was revived once more by

¹ Koch, *American Journal of Physiology*, 1904, xi, p. 312. In a previous paper on the phosphatids, Koch (1902) said that it was unnecessary for him to consider protagon, as he had indicated in that paper the parts of which it was composed—lecithins, kephalins, and cerebrins.

² Hammarsten, *loc. cit.*, 1904, p. 409.

Cramer's (1904) investigations, which were carried out in Liebreich's laboratory. Cramer's results seemed to show that protagon was a definite substance, as originally stated by Liebreich. In a recent review of some problems of physiological chemistry, Chittenden,¹ referring to "the utter lack of agreement among physiological chemists as to the entity of so-called protagon," and influenced, no doubt, by Cramer's paper, concluded that "whether this phosphorized substance (protagon), studied by so many investigators, exists as such in the living tissue, or whether it is simply an intimate mixture of lecithin, cerebrin, and one or more other substances, is not yet settled to the satisfaction of all concerned." Dunham² recently prepared protagon, by Cramer's method, from beef kidneys.

Cramer's observations have necessitated a further investigation of the protagon problem. The experiments described below (page 77) were begun a year ago and have been carried out at intervals since. We believe our results show conclusively that protagon is a variable mechanical mixture. They are in entire accord with the previous conclusions published by Lesem and Gies from this laboratory.

II.—WHAT WAS PSEUDOCEREBRIN?

We raise this question, before beginning the description of our experiments, because Cramer referred to an important matter that had been overlooked by all, apparently, except Thudichum; which bears directly on the work of Wörner and Thierfelder and of Lesem and Gies, and which must be fully understood before we can proceed satisfactorily.

In leading up to his conclusion that "the reasons brought forward by Wörner and Thierfelder and by Lesem and Gies against the chemical individuality of protagon are not conclusive," Cramer observed that Wörner and Thierfelder's cerebrin was merely Gamgee's pseudocerebrin. Cramer believed, therefore, that Wörner and Thierfelder were not dealing with pure protagon when they found the latter to be a mixture

¹ Chittenden, *Popular Science Monthly*, 1904-05, lxxv, p. 162.

² Dunham, *Proceedings of the Society for Experimental Biology and Medicine*, 1905, ii, p. 63.

containing cerebrin and other substances. He also believed that Lessem and Gies worked with protagons that were admixed with pseudocerebrin. Cramer's deduction that pseudocerebrin and cerebrin were identical was undoubtedly correct, but in what respects, it may well be asked, were those products different from Thudichum's phrenosin? We think, however, that the identity of phrenosin, pseudocerebrin, and cerebrin (and the facts that depend upon that identity), instead of justifying the inference of Cramer's that was quoted above, lead directly, as will be indicated below, to a conclusion in harmony with the deduction by Lessem and Gies that "pure" protagon is a mechanical mixture.

PSEUDOCEREBRIN.—Pseudocerebrin was originally prepared and described by Gamgee. Gamgee and Blankenhorn revived the protagon-proposition in 1879. In the only paper on the subject that was contributed by them to a journal devoted to the publication of original contributions, no mention was made of pseudocerebrin. A year later, however, Gamgee (1880) referred in his *Text-Book* to his *unfinished* researches on cerebrin and gave there his previously *unpublished* conclusions on three points, the third group of which were stated as follows:

(3) "In addition to protagon and other phosphorized matters, there is always extracted from brain by alcohol at 45° C., a very considerable quantity of a body, which, in order to distinguish it, the author provisionally termed *pseudo-cerebrin*. This body is less soluble in 80 per cent. alcohol at 45° C. than protagon, so that on subjecting impure protagon to repeated crystallization from 80 per cent. alcohol there accumulated residues consisting of the cerebrin-like body. The latter is a white, pulverulent body, very unlike protagon to the naked eye and separating under the microscope in the form of very large nodular masses. After repeated recrystallization from alcohol it was found to be practically free from phosphorus (containing only 0.08 per cent.). . . . The author refrains from speaking with confidence of the absolute purity of 'pseudo-cerebrin.' . . . It would therefore appear to the author that whilst protagon cannot be separated by the action of solvents into a non-phosphorized cerebrin and a phosphorized body, yet such non-phosphorized bodies exist by its side in the brain, and can be obtained from protagon by the action of caustic baryta" (p. 441).¹

¹ These observations, as was indicated above, were referred to by Cramer, but appear to have escaped all previous authors except Thudichum (1886).

Gamgee has apparently made no further contribution to the subject.¹ It is difficult to understand exactly, from the very brief statement of Gamgee's that is quoted above, what is meant by the direction to subject "*impure*" protagon to "*repeated*" crystallization, for it was shown by Gamgee and Blankenhorn that the composition of ox-brain protagon, which had been recrystallized only *twice* from 85 per cent. alcohol,² was unchanged by a *third* recrystallization, and their results indicated in a general way that protagon from dog-brains that was recrystallized only *once* was as "*pure*" as protagon from horse-brains that was recrystallized *four* times.³ Gamgee and Blankenhorn did not take the subsequently-stated precaution to recrystallize "*repeatedly*," unless only one to "*four or five times*" at most is considered sufficient to meet the requirements of the direction given. Is it not highly probable therefore, quite certain in fact, that their protagons contained pseudocerebrin? Are not the remarks of Gamgee in 1880 indicative of a logical departure from the position held in 1879, and of a feeling that protagon, as previously prepared and described, contained the new substance subsequently discovered by him, *i.e.*, pseudocerebrin?

How can any one know definitely when pseudocerebrin has been completely removed from protagon in the process of recrystallizing from 80 per cent. alcohol at 45° C.? Gamgee has said nothing that will answer the question. He referred, it is true, to the nodular masses in which pseudocerebrin crystallizes, but "*pure*" protagon, that has been recrystallized *ten* times, forms such masses if the solution from which it crystallizes is not too dilute and the crystallization is not too slow (page 105). Is it not almost certain, from the various facts recited above, that every protagon that was described before Cramer drew attention to pseudocerebrin, contained the latter substance? Who of the "*protagonists*" except Gamgee knew about pseudo-

¹ Except that made to Thierfelder and mentioned below (p. 72).

² The different concentrations of alcohol (80 per cent. and 85 per cent.) could not account for the discrepancies noted.

³ "The amount of phosphorus in specimens of protagon which had been crystallized from alcohol *four* or *five* times was not smaller than that present in protagon which had only *once* been crystallized, though a thorough treatment with ether preceded each recrystallization" (Gamgee, *Text-Book*, p. 427).

cerebrin, and who succeeded in removing it from his protagon either by design or accident? If these questions be answered in the negative, and we think they must be, what has protagon been if not always a mechanical mixture of substances?

Was Cramer's protagon assuredly free from pseudocerebrin? If pseudocerebrin was not contained in it, how did Cramer remove it? His methods of preparation and purification warrant the conclusion, however, that pseudocerebrin was present in each of his protagon-preparations. We have separated a pseudocerebrin-like substance from every sample of protagon made by us by the Cramer method (page 94). We believe that the higher temperature prescribed in Cramer's method tends to favor the presence of even a larger proportion of pseudocerebrin in the protagon obtained by means of the new process than that in the protagon prepared by the older and more cautious methods.

What is the effect of the "repeated recrystallization" (necessary, according to Gamgee, to remove pseudocerebrin from protagon), on the protagon itself, or, more correctly, on the phosphorized matter associated with the pseudocerebrin? What is the composition of such associated matter, or of "pure" protagon, if we may call it such? Gamgee gave no information on these points. No one else, we believe, is able to supply a definite answer.

That Gamgee's further studies, unfinished though they were (1880), modified greatly his original views was shown also by his remarks on the great stability of protagon in boiling alcohol¹ and ether, and on the presence in the brain of a *free*, preformed "non-phosphorized cerebrin." His earlier, opposite views have been quoted repeatedly by successive investigators, all of whom, except Thudichum, have remained unacquainted, until recently, with the remarks in the text-book. It is to be regretted that Gamgee's ultimate conclusions on the matters referred to above were not published where they would have been sure to receive the attention they merited. If they had been promptly

¹ On page 426 of his *Text-Book* Gamgee states: "At higher temperatures than 55° C. alcohol appears to decompose protagon" (see footnote, No. 2, p. 99 of this paper). On page 429 the following may be found: "Pure protagon is remarkably rebellious to the action of even boiling alcohol, though that action be continued for hours." Page 440 records the following: "By boiling with alcohol for many hours protagon appears to be decomposed."

published in the journal, for example, in which the paper by Gamgee and Blankenhorn appeared, we believe the protagon question would have been settled before Parcus and succeeding authors could have made the obvious mistakes that have been recorded in abundance since.

PHRENOSIN.—Six years after the appearance of Gamgee's textbook, Thudichum published some results of further experiments that showed anew the indefinite nature of protagon. He also referred (1886) to the changes in Gamgee's views on a number of points and said regarding the altered opinions and related matters:

"Diese Schwenkung war ebenfalls das Resultat meiner Schrift von 1879. Aber ein noch viel wichtigeres Resultat derselben war die Errichtung von Seiten Gamgee's von zwei Categorien von 'Protagon,' der des reinen und der des unreinen. Er sagt, neben 'Protagon' und anderen phosphorhaltigen Substanzen ziehe Alkohol bei 45° C. aus dem Gehirn eine sehr beträchtliche Menge eines Körpers aus, den er einstweilen 'Pseudo-Cerebrin' nennt. Er isolirt denselben mittelst des in meiner Schrift angegebenen Processes der fractionirten Krystallisation aus seinem 'unreinen Protagon' und giebt ihm die Formel $C_{44}H_{72}NO_8$. Dies ist deshalb weiter nichts als mein Phrenosin mit ein wenig Kerasin, welches nicht ganz weggeschafft worden war. Jedenfalls ist 'die sehr beträchtliche Menge' dieses Körpers ein interessantes Object für diejenigen, welche, nachdem sie geraden Weges beim reinen 'Protagon' angelangt sind, nun auf Umwegen den Fortschritt zum 'unreinen Protagon' zu unternehmen gedenken." (Pages 214-215.)

These statements by Thudichum have never before, we believe, been quoted or referred to.¹ Thudichum's method of preparing phrenosin from protagon (or from Vauquelin's "white matter") is indicated in the following quotation from the volume issued by him in 1879:

"The cerebrins are obtained free from phosphorus mainly by very frequent resolution in hot absolute alcohol. They cannot be separated from each other by this process alone; lead acetate and a little ammonia are required to make cerebrinic acid insoluble in hot spirit;

¹ Koch has observed that "Thudichum's phrenosin may be said to be identical or isomeric with the substance isolated by Thierfelder (cerebron) and by Koch (cerebrin)."—*American Journal of Physiology*, 1904, xi, p. 310. Parcus and also Kossel and Freytag noted the resemblances between their cerebrins and Thudichum's phrenosin, but regarded them as different products, nevertheless. Thudichum regarded their cerebrins as practically identical with his phrenosin.

phrenosin and kerasin do not permanently combine with lead, they are separated from each other by fractional recrystallization from suitable volumes of absolute alcohol; phrenosin is deposited earlier at a temperature above 28°C ., kerasin later at temperatures below 26°C ., and on long standing" (p. 262). The method is given in detail in Thudichum's last book (1901).

CEREBRON.—In 1900 Wörner and Thierfelder prepared from protagon a cerebrin-like substance they called cerebron. For its preparation they used a simple method of fractionation in 50 per cent. alcohol-benzol or 50 per cent. alcohol-chloroform at 45° – 50°C ., similar to a number of Thudichum's processes and analogous to Gamgee's method of preparing pseudocerebrin. Cerebron was regarded as an entirely new substance.

A year ago, shortly after the appearance of Cramer's paper, Thierfelder (1904) published the results of a further study of cerebron. He stated, by way of introduction:

"Ehe ich auf die Resultate, welche die weitere Untersuchung dieser Substanz ergeben hat, eingehe, möchte ich nicht unterlassen, mitzuteilen, dass das Cerebron bereits im Jahre 1880 von A. Gamgee beschrieben worden ist. Herr Professor Gamgee teilte mir im Anschluss an eine mündliche Unterhaltung gelegentlich eines Besuches in Berlin im Herbst vorigen Jahres den Wortlaut seiner diesbezüglichen Veröffentlichung mit. Es heisst auf Seite 441 seines Textbook.¹ . . . Herr Gamgee fügte hinzu, dass er bei der Durchsicht seines Laboratoriumsjournals Zeichnungen des mikroskopischen Bildes des Pseudocerebrins, welche mit den von uns veröffentlichten völlig übereinstimmen, gefunden habe, und ferner unter dem 11. Juli 1879, die Eintragung, dass das Pseudocerebrin beim Erhitzen in der Kapillare bei 197° sich leicht orange färbt und bei 210° schmilzt" (pp. 21–22).

Very close agreement of the figures for melting-points and percentage composition of pseudocerebrin and cerebron occasioned further remarks by Thierfelder as follows:

"Darnach kann es nicht zweifelhaft sein, dass Pseudocerebrin und Cerebron identische Körper sind und dass die *Priorität der Entdeckung* A. Gamgee zukommt (see p. 72). Da es nicht üblich ist, neue Beobachtungen nur in einem Lehrbuch zu veröffentlichen, und da das Pseudocerebrin unseres Wissens sich sonst nirgends in der Literatur erwähnt fand,² so dürfte uns ein Vorwurf für dieses Uebersehen nicht treffen" (p. 22).

In the same paper Thierfelder observed that cerebron, on decomposition in hot 7 per cent. sulfuric acid, yielded galactose,

¹ The quotation on page 68 of this paper was given by Thierfelder at this point.

² Cf. with the quotation from Thudichum's account of his experiments.

also a new acid which he called cerebronic acid, $C_{25}H_{50}O_3$ ¹ ("a hydroxyl derivative of an acid of the formula $C_{25}H_{50}O_2$ "), and a base of the formula $C_{17}H_{35}NO_2$. With reference to this base Thierfelder wrote as follows:

"Nachdem die Arbeit bereits druckfertig finde ich, dass Thudichum (1901) schon als Spaltungsproduct seines Phrenosins eine Base von der Zusammensetzung $C_{17}H_{35}NO_2$ isoliert, beschrieben und *Sphingosin* benannt hat. Mit diesem Körper ist offenbar die Base aus dem Cerebron identisch. Ich bin mit der genaueren Untersuchung dieser basischen Substanz beschäftigt und werde bei der Mitteilung der Resultate auf die Angaben von Thudichum eingehen" (p. 30).

It seems singular that Thierfelder did not happen to discover, at the same time and on the same pages of the book of Thudichum's (1901) to which he referred, the fact that cerebron was either identical or isomeric with phrenosin. This was indicated not only by the equivalence of the corresponding figures for elementary composition, but also by the further facts, as stated there by Thudichum, that phrenosin yielded not only sphingosin, but the rest of the decomposition products of cerebron to which Thierfelder referred, viz., galactose and the new acid (or at least one having practically the same composition as cerebronic acid), and called by Thudichum neurostearic acid. The direct results of elementary analysis of the two products and the formulas given them were the following:

	C	H	Formula given.	Composition required by the formula.	
				C	H
Thudichum's neurostearic acid' (1878).....	76.66	12.95	$C_{18}H_{35}O_2$	76.93	12.82
Thierfelder's cerebronic acid (1904).....	75.33	12.50	$C_{25}H_{50}O_2$	75.38	12.56

In his latest contribution on this subject, Thierfelder² referred to the results of a decomposition of cerebron by an improved method—heating with methyl alcohol containing 10 per cent. sulfuric acid. The proportions of the above-named decomposition products were determined. Again there was no reference to the obvious identity of cerebron with phrenosin nor to the significant resemblance between neurostearic acid

¹ Its composition was found to be C = 75.33 per cent; H = 12.50 per cent [O = 12.17 per cent.].

² Thierfelder, *Zeitschrift für physiologische Chemie*, 1905, xliv, p. 366.

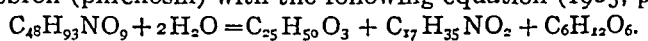
and cerebronic acid. We believe the latter was merely a purer form of Thudichum's product. Thierfelder also refrained from making further direct reference to his confirmation of the essentials of Thudichum's original observations on sphingosin.¹

CEREBRINS.—That Parcus's cerebrin and the cerebrin obtained by Kossel and Freytag were practically identical with phrenosin can hardly be doubted. Koch has referred to the apparent identity of his own cerebrin with Thudichum's phrenosin and Thierfelder's cerebrin, as was indicated in the footnote on page 71.

GENERAL CONCLUSIONS.—The question at the head of this section of our paper, "What was pseudocerebrin?" is correctly answered, we think, by the statement that it was phrenosin. We have also noted the fact that cerebrin and some cerebrins were probably phrenosin. The facts already reviewed are further emphasized by the following figures for percentage elementary composition of the products referred to:

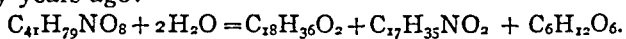
	C	H	N	O
Thudichum's phrenosin (1874).....	69.00	11.08	1.96	17.96
Gamgee's pseudocerebrin (1880).....	68.89	11.87	1.83	17.41
Wörner and Thierfelder's cerebrin (1900)	69.16	11.54	1.76	17.54
Parcus's cerebrin (1881).....	69.08	11.47	2.13	17.32
Kossel and Freytag's cerebrin (1893).....	68.99	11.52	2.25	17.24
Koch's cerebrin (1902).....	68.73	11.83	1.64	17.80

¹ Thierfelder indicated the quantitative relationships of the radicals in cerebrin (phrenosin) with the following equation (1905, p. 370):



Cerebrin	Cerebrin-	Sphingosin	Galactose
(phrenosin)	ic acid		
	(neurostear-		
	ic acid)		

On page 209 of the publication of Thudichum's (1901) to which Thierfelder referred, the same facts are shown by the following equation, which also appeared in various publications of Thudichum's more than twenty years ago:



Phrenosin	Neuro-	Sphingosin	Galactose
(cerebrin)	stearic acid		
	(cerebronic		
	acid)		

The trivial differences between the figures for elementary composition of phrenosin and cerebrin account for the seemingly great differences between the formulas of the original substance that are given above. That Thierfelder's results were more nearly correct may be taken for granted. That the substances were the same seems evident.

In his second paper on cerebrin, Thierfelder, after referring to Gamgee's priority in the discovery of pseudocerebrin, wrote as follows:

"Mit Rücksicht darauf, dass die in Rede stehende Substanz als im Gehirn vorgebildet anzusehen ist, während unter Cerebrinen im allgemeinen Spaltungsprodukte höher zusammengesetzter Gehirnstoffe verstanden werden, halten wir den Namen Cerebrin für zweckmässiger und schlagen vor, ihn beizubehalten" (p. 22).

In view of the facts recited above it seems to us that the name applied by Thudichum, who discovered pseudocerebrin, cerebrin, and the several cerebrins, should be retained and applied to all the products referred to in the above summary, until there is good reason to think any of them is not the same as phrenosin. We see nothing objectionable in the term phrenosin. We believe also that phrenosinic acid would be a more acceptable name for what Thudichum termed neurostearic acid and Thierfelder called cerebronic acid.

III.—WERE THE "PURE" PROTAGONS ANALYZED BY LESEM AND GIES MIXTURES OF PROTAGON AND PHRENOSIN?

We quote the following remarks from the introduction to Cramer's descriptions of his experiments:

"By treating protagon, the analysis of which gave figures corresponding to other authors', with dilute alcohol for several times, Lesem and Gies got substances which differed widely in their percentage of phosphorus and in their solubility in alcohol. The table given by them (p. 192) will explain their method and results. The decreasing percentage of phosphorus in the 'precipitated protagon' corresponds entirely with the statement of Gamgee regarding pseudocerebrin. We may therefore assume that the 'protagon' of Lesem and Gies is a mixture of protagon and pseudocerebrin. Indeed, the precipitate of the first extract, where the protagon should be purest, has a percentage of phosphorus equal to that of protagon. It remains to explain how it is that this mixture of a phosphorus-free with a phosphorus-holding substance should on analysis give a percentage of phosphorus corresponding with that of protagon, when one would, of course, expect a lower figure. This deficit must have been made up by a substance richer in phosphorus than protagon itself. This substance is indeed present in Lesem and Gies's protagon, for in the filtrate from the freshly precipitated protagon of the first extract and in the alcohol-ether washings we find a substance containing a higher percentage of phosphorus" (pp. 32-33). The summary given by Lesem and Gies is presented at the top of the next page.

FRACTIONAL PRODUCTS OF PROTAGON OBTAINED BY LESEM AND GIES.

Fractional Product.	Weight in Grams. ¹	Percentage of Phosphorus.
A. Freshly precipitated protagons:		
a. From first extract.....	10.834	1.23
b. From second extract.....	7.599	0.89
c. From third extract.....	1.729	0.57
B. Insoluble protagon (residue).....	2.009	0.12
C. Substance in filtrates from the freshly precipitated protagons:		
a. Of first extract.....	0.785	2.59
b. Of second extract.....	0.678	1.31
c. Of third extract.....	0.250	0.85
D. Alcohol-ether washings of the freshly precipitated protagons.....	0.282	2.02
Substance recovered.....	24.17	—
Substance taken.....	24.34	1.16

We have already indicated (page 70) the probability that all preparations of protagon that were made previous to Cramer's work contained phrenosin. On the same page it was stated that we obtained phrenosin-like products from every sample of protagon made by us by Cramer's method, that we examined (see page 94). The fact expressed in the last sentence of the above quotation from Cramer's remarks on the results obtained by Lesem and Gies must also be explained away satisfactorily before it will be possible for any one to insist that protagon prepared by the classical methods in general use before the appearance of Cramer's paper was not always a mixture. Cramer prepared protagon by a new method but did not investigate the question whether protagon was a mixture. He did not subject his new protagon to a fractionation process and consequently left untouched the fundamental point alluded to in the last sentence in the remarks of his we have quoted.²

Suppose it be admitted that Lesem and Gies, and all other protagon-investigators to the time of Cramer's work, analyzed

¹ The weights are for substance dried to constant weight in vacuo over H_2SO_4 .

² Dr. Cramer informed us that the experimental part of his work was completed before the publication of the paper by Lesem and Gies, and that it was only lack of proper opportunity to take up the work again that prevented him from going into this matter.

products containing phrenosin (pseudocerebrin, cerebrin), and we think as indicated before that this must be conceded, would it not also be admitted at the same time that the idea contended for so long unavailingly by Thudichum, and recently by Lesem and Gies, that protagon was a mixture, was correct? But what was the nature of the phrenosin-free part of the mixture that has always been called protagon? If now we decide to apply, as Cramer does after Gamgee, the name protagon to the phrenosin-free part of the old protagon, do we not merely shift our point of view and give to a part the name that has heretofore been applied to the whole?

Did any one ever succeed in separating and identifying this hypothetical protagon—the part of protagon that would remain if phrenosin were removed from it? If we admit Cramer's conclusion that Lesem and Gies were dealing with mixtures of such *hypothetical* protagons and phrenosin, then surely we are also obliged to admit that it was this newly conceived protagon in their mixtures that yielded the products of relatively high and distinctly *variable* phosphorus-content that were alluded to in the quotation above.¹ But if Cramer's view were correct would not this hypothetical protagon also have to be conceived as a mixture? It stands to reason also that after removal of the phrenosin from the protagon-mixture, the phosphorus-content of the remainder would be much higher than any ever before recognized as typical of protagons.

We have prepared pure protagon by Cramer's methods and have subjected samples of the products thus obtained to various fractionation processes that are described below (page 94).

IV.—PROTAGON PREPARED BY CRAMER'S METHOD.

THE METHOD.—The following statement was made by Cramer as an introduction to the description of his new method:

"The fact that so many different observers have been able to extract from the brain a substance of uniform composition—the protagon—has been explained by Thudichum by the fact that they have all endeavored to observe the same conditions and to avoid any very energetic procedure,

¹ The products of high phosphorus-content could not have been impurities, because the methods of Lesem and Gies and their analytic results furnish conclusive evidence against that possibility.

as, for instance, dissolving in boiling alcohol. In order to investigate the question as to the existence of protagon, I tried to prepare it by a different method. For this purpose I made use of the observation, kindly communicated to me by Professor Liebreich, that protagon is coagulated by boiling it with salt solutions." (P. 33.)

Cramer did not indicate any special advantage to be gained by the use of this coagulation method except that it was different from any before employed. In the original paper on protagon, Liebreich referred as follows to the coagulating effect mentioned by Cramer:

"Behandelt man das reine Protagon mit Wasser, so quillt es ungemein stark auf und stellt eine undurchsichtige kleisterartige Masse dar. Verdünnt man mit mehr Wasser, so erhält man eine zwar klare aber doch opalisirende Lösung. Mit concentrirten Lösungen der Salze, wie Chlorcalcium, Chlornatrium u. s. w., gekocht tritt eine Coagulation ein. Die in der Flüssigkeit herumschwimmenden Flocken von Protagon lassen sich abfiltriren, so dass in dem Filtrat kein Protagon mehr nachgewiesen werden kann. Das Coagulum besteht aus keiner chemischen Verbindung, da sich auf dem Filter die Salze wieder auswaschen lassen und das Protagon in dem Masse aufquillt, als die Salze ausgewaschen werden. Jedoch ist es schwierig, auf diese Weise es wieder vollständig rein zu erhalten, weil die Substanz, je mehr sie ausgewaschen wird, sich löst und somit durchs Filter geht." (Pp. 33-34.)

The method employed by Cramer was given as follows:

"Brains freed from the membranes and finely minced were pressed through a sieve together with a hot solution of sodium sulfate, and then heated in the water-bath at 100° C. till the brain substance coagulated, and left a clear fluid at the bottom of the flask, which was siphoned off. The remaining brain substance was treated with a fresh solution of sodium sulfate once more in the same way. The brain substance, freed as much as possible from the salt solution, was then extracted in the water-bath as before, with 95 per cent. alcohol for the first time, followed by extraction with 85 per cent. alcohol, till on cooling nothing was deposited. The filtrate was cooled to 0° C., then filtered through a funnel cooled with ice-water, and the precipitate recrystallized out of boiling 85 per cent. alcohol. In order to free the substance from cholesterin, it was treated with ether, at first in the flask and in the cold, till all the alcohol had been removed, then in the Soxhlet apparatus, until the Liebermann-Burchard test for cholesterin gave a negative result. The residue was dried *in vacuo* over P₂O₅, twice recrystallized out of boiling absolute alcohol, and again dried over P₂O₅. The protagon thus prepared was a snow-white, pulverulent powder, which did not suffer change on exposure to the air. The material used was obtained from ox-brains. Usually two brains were worked up together, giving 5-7 gr. of dry protagon." (P. 33.)

In response to our request for certain minor details connected with his method, Dr. Cramer, who has favored us with encouraging expressions of hearty interest in our research, kindly gave us the following further information (letter, October 8, 1904):

"I used to make up a solution of 100 g. of sodium sulfate in 2000 c.c. of water, heated to ca. 60° C. The two minced brains were suspended in ca. 300 c.c. of the solution and pressed through the sieve. Then ca. 500-600 c.c. of the solution were added and the whole heated in the water-bath. As a rule, the brain substance coagulated after a short time (5-10 minutes), leaving a clear fluid at the bottom of the flask. If the fluid was not clear I added more sodium sulfate solution. The treatment was repeated with about 600 c.c. of the solution. On the average I used 1500 c.c. of the solution. When I recrystallized the substance, which had been extracted by alcohol from the coagulum, out of 85 per cent. alcohol, a considerable amount of this substance remained undissolved. I refrained from apparently increasing the production of protagon, and did not add more alcohol than was necessary to dissolve that part which is more easily soluble."

These additional details enabled us, we believe, exactly to repeat Cramer's method,² which we did with the following results:

PROTAGON-PREPARATIONS 1-8 (SHEEP-BRAINS).—One hundred and fifty sheep brains were heated with 16 liters of the sulfate solution. The

¹ The latter precaution *reduced* the amount of phrenosin (pseudocerebrin) that tended to go into solution with the phosphorus-containing portion of the crude product. It could not have *entirely* prevented solution of the contained phrenosin, however, for the solubility of the latter in alcohol is increased by the presence of the phosphorus-containing material, and the latter favored solution of some of the former on each successive treatment with alcohol.

² Aside from dehydrating the tissue to a certain extent and removing phosphates, other salts and various substances soluble in alcohol also, we fail to see any particular advantage possessed by the preliminary part (new) of this method. The usual impurities of protagon, such as cholesterolin, do not appear to be removable by the process of treating the tissue with hot salt-solution. It is possible, on the other hand, that the preliminary treatment is disadvantageous, for the coagulated protagon may be harder to extract from the tissue than the uncoagulated material would be. Liebreich alluded to the difficulty of removing from the protagon the salt used in coagulating it. We have not yet learned what the "coagulation" of protagon actually is, although the effect appears to be identical with related phenomena in various colloidal solutions under similar conditions.

maximum temperature attained on the first treatment was 96° C., on the second it was 86° C. Our material had been so finely minced at the beginning that very much of it was carried with the fluid when we endeavored to separate the latter by straining it through cheese-cloth. The fluid was therefore removed from the tissue by decantation. The tissue was thoroughly washed by the same means with cold 5 per cent. sodium sulfate solution, and also twice with small amounts of 50 per cent. alcohol, under a fresh portion of which it was allowed to stand overnight. In this way most of the sulfate was removed, although a large proportion of it remained in the tissue. The latter was finally separated by filtration. About 10 liters of alcohol were used at a time for extracting the protagon. Eight extractions in a total of 80 liters were necessary to remove all the substance that separated on cooling. The several extracts were kept for about 36 hours in a large refrigeration room, where the temperature was maintained steadily at -3° C. The precipitates were filtered off rapidly on a suction apparatus. Each product was washed free from cholesterin with cold ether *before* recrystallization from hot 85 per cent. alcohol. The recrystallized product consisted of rosettes of needles. It was finally washed again with fresh, cold ether before it was dehydrated in the desiccator. The properties of the products were apparently identical with those of the preparations described by Cramer. Our quantitative data are summarized in Table I.

TABLE I.

PROTAGON-PREPARATIONS MADE FROM SHEEP-BRAINS BY THE CRAMER METHOD.

	1	2	3	4	5	6	7	8	Combined 1-8.
Grams of crude product ¹	20.0	49.7	30.8	25.6	22.6	11.1	6.1	0.9	166.8
Grams of purified product.....	13.8	44.1	25.3	20.3	19.2	8.3	3.4	0.4	134.8
Percentage composition of purified product: ²									
Phosphorus.....	0.90	1.07	1.03	0.95	0.92	0.94	0.90	0.89	—
Nitrogen.....	—	2.07	—	2.02	—	—	—	—	—
Sulfur.....	—	0.62	—	—	—	—	—	—	—
Ash ³	4.31	3.95	—	—	—	—	—	—	—

¹ After removal of cholesterin by washing with ether.

² Phosphorus and sulfur were determined, here and in the rest of the experiments, by the usual fusion methods. Nitrogen was determined in all our work by the Kjeldahl method—oxidation was effected with concentrated H₂SO₄ aided by CuSO₄.

³ The ash dissolved in water and formed an acid solution (P₂O₅), which, on further acidification with HCl, gave only a very faint turbidity with BaCl₂, but yielded a heavy precipitate with molybdic solution.

General Observations.—These observations indicate the difficulty of removing the last portions of protagon from brain (see page 103). The differences between the amounts of crude protagon and the corresponding quantities of purified products were due almost entirely to the removal of matter that remained insoluble on preparing the solution for recrystallization of the protagon, plus the substance that remained in solution after the protagon had been reprecipitated by cooling (see page 82). The data for phosphorus, nitrogen, and sulfur make it evident that our preparations were practically the same in quality as Cramer's samples A, B, and C, although the latter were prepared from ox-brains. This fact agrees, however, with previous observations that protagon-preparations from the brains of different mammals were always practically the same. The variation in the figures for content of phosphorus, trifling though it is, harmonizes with the idea that each product was a mixture that differed slightly from the rest.

PROTAGON-PREPARATIONS 9-12 (OX-BRAINS).—In a second series of preparations of protagon made by Cramer's method, as outlined above, we used ox-brains in order to further examine the process and its results. Ten ox-brains were taken. The maximum temperature attained in the treatment with sulfate solution was 95° C. Washing with 50 per cent. alcohol was omitted, because the fluid could easily be removed this time through cheese-cloth without appreciable loss of solid matter. The latter was thoroughly and rapidly washed by decantation three times in water—14 liters each time. Most of the sulfate was removed in this way. The washings became opalescent, but only faintly so. Five extractions were made with alcohol in volumes of 5 liters at a time. The remaining details of procedure for the previous preparation were applied to this one. Filtration of the first two precipitated extracts was comparatively difficult, apparently because of the larger proportions of cholesterol in them.¹ The original alcoholic filtrates, with the first ether-washings from the corresponding protagon-precipitates, were evaporated to dryness on a water-bath and the residues weighed. Our quantitative data are given in Table II (page 82.)

General Observations.—The variation of phosphorus-content in the second series of protagons is similar to that of the first series and more indicative of the fact that protagon is a mixture of substances. The products were practically the same as Cramer's, as every examination of their properties showed.

¹ We have found that protagon is more easily obtained from sheep-brains than from ox-brains.

The proportion of soluble matter in the several filtrates from the protagons was relatively large, although a portion of each amount consisted of unidentified ether-soluble matter.¹

These observations were in accord with Cramer's conclusion that typical protagon may be prepared by the new method.

TABLE II.

PROTAGON-PREPARATIONS OBTAINED FROM OX-BRAINS BY THE CRAMER METHOD.

	9 ²	10	11	12	Combined 9-12.
Grams of purified protagon.....	17.7	19.9	13.9	3.8	55.3 ³
Percentage composition of purified protagon:					
Phosphorus.....	1.04	0.93	0.97	0.77	—
Nitrogen.....	2.35	—	—	—	—
Sulfur.....	0.75	—	—	—	—
Grams of solid matter in the original alcoholic filtrates and corresponding ether-washings ⁴	3.76	3.36	2.16	0.84	10.12

V.—A STUDY OF THE EFFECTS OF CRAMER'S METHOD ON PROTAGON PREPARED FROM SHEEP-BRAINS BY THE GAMGEE AND BLANKENHORN PROCESS.⁵

The original observations by Liebreich that were quoted on page 78 indicate that the process of heating protagon in neutral salt solutions exercises merely a physical effect upon it. Cramer has apparently taken it for granted, with Liebreich, that the process referred to does not result in any chemical transformations of the protagon. This matter was investigated in the following experiments, in which we also prepared a number of protagons for fractionation by the methods indicated on page 94.

¹ Not cholesterin, for that had been completely removed previously (see page 109).

² The protagons of the first and second extracts were combined.

³ About the same proportionate yield as Cramer's.

⁴ Phosphorus-proportions in similar products are given on page 96.

⁵ This well-known method was selected for the preparation of pure protagon to be used in our comparative studies, because the process at once superseded Liebreich's and has since been universally regarded as the best.

PREPARATION OF CRUDE PROTAGON-PRODUCTS BY THE GAMGEE AND BLANKENHORN METHOD.—The method as originally described by Gamgee and Blankenhorn (p. 277) and again in Gamgee's text-book (p. 427) was followed in detail. All of the crude products referred to below were washed free from cholesterin with large volumes of cold ether and dried in desiccators over concentrated sulfuric acid. The following data were recorded:

Crude product A.—Fifty sheep-brains were extracted twice in 16 liters of alcohol at a time. The first extract was made in five hours, the second in twenty-four.¹ The extracts were kept in the refrigeration room (-3°C.) thirty-six hours. The precipitates consisted almost wholly of spheres, nodular masses, and platelets of cholesterin. The product from the second extract was greater in quantity than that from the first. The preparation consisted of waxy lumps with yellowish tinge, which could be easily converted to a light powder. Total yield, 22.8 grams.

Crude product B.—One hundred sheep-brains were extracted in two volumes of 24 liters each of alcohol. The remaining details of procedure were the same as for crude product A, with the same qualitative results. Total yield, 17 grams.

Crude product C.—The conditions attending the preparation of product B were duplicated except that each extraction was continued about twenty-four hours. The product from the first extract was chiefly cholesterin. The characters of the final product were essentially the same as those of product B, except that there was scarcely any suggestion of a yellowish tinge. Total yield, 22.5 grams.

Crude products D and E.—The conditions attending the preparation of products B and C were duplicated, except that the first extract was made in five hours and discarded because it contained only a trifling quantity of protagon. A third extract was made that proceeded for nearly twenty-four hours. The extracts were kept in the refrigeration room forty-eight hours. The protagons of the second (D) and third (E) extracts were treated separately. Each consisted of globular forms; needles were missing. Each product was snow-white, and consisted of waxy lumps that could be ground easily to fine light powders. Product D was found to dissolve, somewhat more readily than E, in 85 per cent. alcohol at 45°C. Separate yields: D, 17.8 grams; E, 48 grams. Total yield, 65.8 grams.

Crude products F, G, H, and I.—The conditions of preparation of products D and E were duplicated. Five alcoholic extracts were made, with results qualitatively the same in detail. Separate yields: F, 12.8 grams²; G, 27.2 grams; H, 34.3 grams; I, 47.0 grams.³ Total yield, 121.3 grams.

¹ It was soon found that two extractions were not sufficient for a good yield of protagon.

² The first extract was discarded as before.

³ Further extraction would have been necessary to remove all of the

PURIFICATION OF CRUDE PROTAGON-PRODUCTS PREPARED FROM SHEEP-BRAINS BY THE GAMGEE AND BLANKENHORN PROCESS. *Protagon-preparations 13 and 14.*—Crude products A, B, and C (a total of 62.3 grams) were each treated separately twice with about two liters of 85 per cent. alcohol (a total of 12 liters) at 45° C. The first extractions (yielding, when combined, protagon 13) were continued five hours; the second extractions (yielding, when combined, protagon 14) were made in fifteen hours. A yellowish residue from each fraction persisted to the end of the alcoholic treatments. In each case it was soft in the warm alcohol, but hardened on cooling. The first extracts were kept in the refrigeration room twenty-four hours; the second, twelve hours. The two protagon-products and the combined residues were partially dehydrated¹ with cold ether overnight and then dried in the usual manner. The preparations of protagon were typical in all physical qualities. The residue was easily powdered. The powder was brownish white. Quantitative data are given below:

TABLE III.

	Protagon.		Insoluble Matter.	Total Weight of Substance Involved:		
	13	14		Taken.	Re-covered.	Lost in Filtrates and Washings. ²
Weight in grams.....	21.0	14.5	2.8	62.3	38.3	24.0
Percentage of phosphorus...	1.73	0.89	0.54	—	—	—

protagon, of which, it was evident, a considerable amount still remained in the tissue (see p. 81). It might be presumed, judging from Gamgee's and Cramer's remarks on phrenosin (pseudocerebrin), that by this *partial* extraction, protagons of relatively high phosphorus-content would be obtained (see p. 68).

¹ Cholesterin had previously been completely removed from the crude products.

² The proportion of substance lost in such treatment depends not only upon the "purity" of the protagon, but also on the volumes and temperatures of the solvents and wash-liquids (see pp. 92 and 99).

³ In the upper footnote on this page it was suggested that our method of *partial* extraction of the protagon from brain instead of *complete* extraction, as is usually the case, might be expected to give protagons of relatively high phosphorus-content (see p. 75). The duplicate results on which the above figures depend were 1.77 per cent. and 1.69 per cent. The alcohol employed in all our experiments was freshly distilled and entirely free from phosphorus. See the succeeding experiments and remarks on p. 87.

Protagon-preparations 15 and 16.—Crude products D, E, and F (a total of 78.6 grams) were combined and extracted twice with two volumes (12 liters each) of 85 per cent. alcohol at 45° C. The first extract (yielding protagon 15) was made in six hours; the second (yielding protagon 16) was carried out in twelve hours. A soft white residue persisted to the end. Refrigeration of each was maintained twenty-four hours. The dry residue was waxy and white, and yielded a light white powder. The protagons were typical. Further data are subjoined:

TABLE IV.

	Protagon.		Insoluble Matter.	Total Substance Involved:		
	15	16		Taken.	Re-covered.	Lost in Filtrates and Washings.
Weight in grams.....	25.5	20.5	7.3	78.6	53.3	25.3
Percentage of phosphorus.....	1.71 ¹	1.24	0.62 ²	—	—	—

Protagon-preparations 17 and 18.—Crude products G, H, and I (a total of 108.5 grams) were combined and subjected to essentially the same process as that used for the preparation of protagons 15 and 16, with identical qualitative results. The exceptions in treatment were that the first extract (protagon 17) was obtained in twelve hours, the second (protagon 18) in six hours—just the reverse of the lengths of the preceding periods; and refrigeration was carried on for only twelve hours. Additional data are given in the appended summary:

TABLE V.

	Protagon.		Insoluble Matter.	Total Substance Involved:		
	17	18		Taken.	Re-covered.	Lost in Filtrates and Washings.
Weight in grams.....	44.5	22.3	15.0	108.5	81.8	26.7
Percentage of phosphorus.....	1.59 ³	0.90 ⁴	0.74	—	—	—

General Observations.—The figures for phosphorus-content of the first of each pair of protagon-preparations (13–18) from the crude products (A–I) are very striking. They exceed any similar figures heretofore given for brain protagon—and are for

¹ The duplicate results were 1.74 per cent. and 1.68 per cent.

² The duplicate results were 0.60 per cent. and 0.63 per cent.

³ The duplicate results were 1.60 and 1.58

⁴ The duplicate results were 0.88 and 0.92.

protagon *once recrystallized* too!¹ The crystals were typical in each case: needles—isolated, bent, straight, and in rosettes; also some spheres. In one of his preparations of protagon (human brain) Liebreich found 1.5 per cent. of phosphorus, the highest previously recorded for a brain-protagon,² but it has generally been assumed that that result was due to analytic error.³ Kossel obtained protagon with a phosphorus-content of 1.35 per cent.

Each of the protagons prepared from the crude products was obtained before we began the analyses or we should have made certain variations of treatment that have occurred to us since our figures were obtained, in order to investigate this matter more closely. We believed, at first, that two regularly employed modifications of the usual procedure accounted for the high content of phosphorus noted, viz., long-continued refrigeration in alcohol at -3° C. (12 to 48 hours)⁴ for precipitation of the protagons, and long-continued treatment with large excesses of ether at -3° C. (12 to 24 hours) for removal of the main bulk of admixed cholesterin. It was thought that under these conditions the proportions of substance or substances high in phosphorus-content, which Lesem and Gies separated from protagon, were increased in the crude products as well as in each, especially the first, of the "purified" fractions, including the residues. As our work progressed, however, this view lost its probability, because the same treatment of other products did not give rise to the unusual results noted. Another explanation now appears more reasonable, as was indicated in the footnote on page 84.

The above preparations (13-18) were made from crude pro-

¹ "The amount of phosphorus in specimens of protagon which had been crystallized from alcohol four or five times was not smaller than that present in protagon which had only once been crystallized."—Gamgee (*Text-Book*, p. 427).

² Couerbe's results are disregarded (p. 63).

³ Dunham has recently prepared, from beef *kidneys* by the Cramer process, protagon having 2.19 per cent. of phosphorus (*Proceedings of the Society for Experimental Biology and Medicine*, 1905, ii, p. 63)

⁴ Under ordinary circumstances of refrigeration (3 to 4 hours at 0° C.) substances of high phosphorus-content were found by Lesem and Gies in every cold protagon-filtrate they examined. We have obtained the same results.

ducts that had been separated in each case from the first few members of an *incomplete* series of alcoholic extracts of brain. The most easily soluble constituents of the protagon-mixture are doubtless always most conspicuous in the first brain-extract of a series and least abundant in the last of such a series. Phrenosin (pseudocerebrin), we are told, is less soluble, in the usual extractant, than what accompanies it in protagon. and protagon may be freed from phrenosin by avoiding the use of an excess of the solvent. It is just as probable that the greater the number, volume, or temperature of the extracts made of the brain, the larger would be the proportion of phrenosin in the total mass of crude protagon thus obtained and the lower would be the phosphorus-content of the latter. The converse of the statement as to proportionate content is true, we know very definitely, of the more soluble cholesterin. By only *partially* extracting protagon from the brain, as was the case in preparing the crude products (A-I), we obtained chiefly the more readily soluble substances, *i. e.*, those of high phosphorus-content commonly present in smaller proportions in protagon—in a manner analogous to, but more emphatic than, the separation of similar fractional products from *pure* protagon by Lesem and Gies and recently also by us (p. 94). Conversely, the more completely the brain is extracted the larger becomes the proportion of phrenosin that the combined protagon-product contains and the more closely it conforms in phosphorus-content to "pure" protagon—because "pure" protagon has always been prepared from "complete" extracts. The highest content of phosphorus (among the first of each pair of protagons referred to—13, 15, and 17) was evidenced in the first protagon (13) separated from crude products (A, B, and C), comprising the protagon (only 62.3 grams) from the *first two (very incomplete)* extracts of each of three lots of sheep-brains (a total of 250). If this had not been true, our explanation of the cause of high phosphorus-content could not be valid. In further perfect harmony with our conclusion, the *lowest* content of phosphorus in the three protagons referred to was shown by the first protagon (17) separated from crude products (G, H, and I), representing the protagon (108.5 grams) from the *last three* (much more extended, though still only partial) extracts of a series of five from the same lot of sheep-brains (100).

The views expressed on the previous page were not materially strengthened by the facts connected with the preparation of protagonists 1-12 by the Cramer process, but although the conditions of these experiments were not directly comparable to those in which protagonists 13-18 were prepared, especially because of the higher temperature¹ employed in making the extracts and recrystallizing the protagonists,² the decline in content of phosphorus from the protagonist of the first extract to the last does seem to bear out, to a certain extent even there, the conclusion just noted.

We believe that protagonist is such a variable mixture that exceptional circumstances of preparation or of combination of phosphatids and related products in the brain may cause such variations as those noted. We are very confident our analytic data in these experiments were entirely correct and that the above results are among the new ones adduced that show the variability and indefiniteness of the protagonist mixture.

The results referred to above led us to ascertain temperature-effects on protagonist and to study the influence on it of volume of solvent and further washing with ether. The experiments described on page 99 show that the composition of protagonist changes remarkably with variations of the temperature at which it is deposited from its solutions. See page 92 for results on influence of volume of solvent, and page 90 for effects of continuous extraction with ether.

The fractional results shown above are in harmony with the fractional results published by Lesem and Gies, although we cannot lay special stress upon them, because crude products, *i. e.*, unrecrystallized products, were used in the first place (see page 94). It is clear, however, that these results are opposed to the idea that protagonist is something stable or definite, for each protagonist of each pair (13-18) might be called "protagon once recrystallized." Yet which of each pair is protagonist? Which is impurity? What standard can be used to determine the answers to these questions? (Page 69.)

¹ See the experiments described on page 99.

² Which resulted in prompt solution of phrenosin in relatively larger quantity.

PROTAGON PREPARED BY THE GAMGEE AND BLANKENHORN METHOD, AND SUBSEQUENTLY TREATED BY THE CRAMER PROCESS. *Protagon 19.*—Seventeen grams of protagon 13 were treated by the Cramer process as if the material had been brain-tissue; 500 c.c. of sulfate solution were employed. The protagon became soft and somewhat pasty, like starch in hot water, and the stirred mixture was milky. The filtrate was only slightly opalescent. Filtration could not be hastened because of the viscid character of the contained particles, and was allowed to proceed slowly to completion overnight. The solid white protagon obtained by filtration was repeatedly extracted with 200 c.c. of hot 85 per cent. alcohol¹ until an extract (eighth) failed to become turbid when cooled.²

Each extract was cooled separately. The usual crystalline forms of protagon were observed in each product. Spheres and nodular masses appeared in only the earlier extracts. Rosettes of needles and chrysanthemum forms were especially conspicuous in the later extracts. The ninth extract gave no turbidity on cooling and was discarded. A slight residue, apparently consisting chiefly, if not wholly of sodium sulfate, remained after the ninth extraction. The eight turbid extracts were finally combined and kept in the refrigeration room 24 hours. After washing with ether and drying as usual, 15.7 grams were recovered. This dry product was further washed with 4 liters of 85 per cent. alcohol at 0° C., again with cold ether and finally dried as usual, when 14.5 grams were recovered—a total loss of 2.5 grams.

General Observations.—The phosphorus-content of the final product was 1.51 per cent. The phosphorus in the protagon (13) from which it had been made was 1.73 per cent. This difference was no greater than that ordinarily brought about by the *complete* solution of a given mass of protagon and its re-crystallization. A small amount of substance of relatively high phosphorus-content *always* remains in solution under such circumstances (p. 96), and the content of phosphorus in the new precipitate is lowered. This statement is dependent on the results previously obtained by Lesem and Gies and on our own data (page 109). The high content of phosphorus in the protagon obtained after application of the Cramer process strengthens the conclusion that protagon 13 was a mixture of unusual proportions, though typical protagon nevertheless.

¹ The small amount of moisture present made initial treatment with 95 per cent. alcohol undesirable.

² Our purpose here was not to prepare protagon containing less phrenosin than before, but to ascertain directly the effect of the Cramer process on the entire mass of protagon.

Protagon 20.—The preceding experiment was repeated : 23 grams of protagon 15 were treated with 600 c.c. of the sulfate solution. The filtrate was somewhat more opalescent than before.¹ Five extractions of the soft white protagon-mass were made with as many volumes of 200 c.c. of 85 per cent. alcohol. The fifth extract did not grow turbid on cooling and was discarded. The crystalline characters of each deposit were practically the same as those of the previous experiment. A slight residue of sulfate remained. The total yield of ether-washed dry product was 22 grams. After a special washing of the dry product in 4 liters of cold 85 per cent. alcohol and subsequently with cold ether once more, 21 grams were left—a loss of only 2 grams. The content of phosphorus in this product was 1.55 per cent. The phosphorus of the protagon from which it had been prepared (15) was 1.71 per cent.

The conclusions drawn at the end of the description of the preceding experiment apply with equal pertinence to the data of this experiment.

Protagon 21.—The preceding experiment was exactly repeated with 13 grams of protagon 14, having a content of phosphorus a little lower than that of the average protagon-product—0.89 per cent. 11.5 grams were ultimately recovered (a loss of 1.5 grams), in which the amount of phosphorus was 0.83 per cent.

The results of the last three experiments show conclusively that protagon, as prepared by the Gamgee and Blankenhorn method, is practically unaffected by the Cramer process. They further indicate, in harmony with the conclusions on page 82, that protagon made by the latter method is essentially the same as that prepared by the former.

VI.—INFLUENCE ON PROTAGON OF LONG-CONTINUED EXTRACTION WITH ETHER.

The unusually high results for content of phosphorus in protagons 13, 15, and 17 led us to determine the influence of large quantities of fresh ether at ordinary temperatures on our products. Although washing with ether had been very thorough during the process of preparation, in order to remove especially

¹ This filtrate was evaporated to dryness at 40° C., and the residue extracted with about 50 c.c. of warm 98 per cent. alcohol. This extract remained perfectly clear on cooling. The amount of protagon in the filtrate was therefore inconsiderable.

all cholesterin, it seemed possible that other ether-soluble matter, such as lecithin with high phosphorus-content, had not been washed out completely. Gamgee and others observed, in opposition to Diaconow and Thudichum, that phosphorus-containing substance could not be washed out of "pure" protagon with ether at ordinary temperatures. We ascertained the facts regarding our own preparations as follows:

Dry samples of protagons 13, 16, and 20 were subjected to continuous extraction with pure anhydrous ether. Each sample was under ether in a Soxhlet apparatus for a month. The ether was gradually renewed by distillation daily during periods of about 10 hours. The quantitative results are summarized below:

TABLE VI.

Protagon Taken:			Protagon Recovered:	
Number of Preparation.	Amount. Grams.	Phosphorus-content %	Amount. Grams.	Phosphorus-content %
13	2.97	1.73	2.75	1.69
16	1.64	1.24	1.41	1.22
20	1.81	1.55	1.62	1.56

The uniform loss of about 0.2 gram of substance was due to mechanical difficulties in the manipulations; in part, also, to more perfect dehydration of the final products. A small amount of the protagon also actually dissolved. The temperature of the ether surrounding the substance was never high enough to exert marked solvent action however. The maintenance of the high contents of phosphorus in protagons 13 and 20 adds further significance to the remarks made on page 87.

It is obvious that purified *dry* protagon, *i. e.*, protagon that has been thoroughly washed free from cholesterin with cold ether, no longer contains phosphorized substance specially soluble in ether. "Pure," freshly precipitated (hydrated ?) protagon yields to warm ether a considerable quantity of substance that contains less phosphorus than the protagon itself (page 109). The above results show that we succeeded by the

purification process in thoroughly washing our protagonists free from ether-soluble matter.

Even long-continued boiling of "pure" dry protagonist in ether was without effect on its phosphorus-content.

Thus, 7.3 grams of dry protagonist 5, containing 0.92 per cent. of phosphorus, were placed in a flask with reflux condenser, containing 500 c.c. of anhydrous ether, and the mixture boiled gently over a water-bath 17 hours daily for two days. At the end of that time the mixture was rapidly filtered at or near the boiling temperature. On cooling, the filtrate became faintly turbid immediately and a slight amount of flocculent matter was deposited. The weight of dry protagonist recovered was 6.23 grams with a content of phosphorus equal to that of the original material—0.92 per cent.

VII.—INFLUENCE OF VOLUME OF SOLVENT ON THE PROPORTION OF PHOSPHORUS IN REPRECIPITATED PROTAGONS.

Having found to our entire satisfaction that protagonist prepared by Cramer's method was practically the same as protagonist prepared by the Gamgee and Blankenhorn process, we endeavored to ascertain directly whether it could also be fractionated into dissimilar parts by the method used by Lesem and Gies. Before applying the latter process, however, we determined what no one seems to have inquired into, viz., the influence of volume of solvent on the proportion of phosphorus in reprecipitated protagonists. Whatever that influence might be would doubtless be exerted in fractionation experiments such as those of Lesem and Gies.

We proceeded as follows: 14.8 grams of protagonist 16, containing 1.24 per cent. of phosphorus, were treated with 825 c.c. of 85 per cent. alcohol at 45° C. for 24 hours; 4.5 grams failed to dissolve. The solution contained 10.3 grams of protagonist. At the end of the warming process, the solution was made up exactly to 825 c.c., when 1 c.c. contained 0.0125 gram of protagonist. All parts of the filtration apparatus and the receiver were kept at 45° C. so as to prevent deposition of protagonist. Equal volumes of the warm, perfectly clear filtrate were diluted with different volumes of 85 per cent. alcohol at 45° C. The thoroughly mixed solutions were allowed to stand at room temperature overnight, and then were taken to the refrigeration room and kept at -3° C. for 24 hours. The precipitates were rapidly filtered off, and washed and

dried as usual. The filtrates, combined with the ether-washings, were evaporated to dryness. The subjoined summary gives our quantitative data:

TABLE VII.

No.	Solutions.			Dry Protagon.		Phosphorus-content of recovered protagon. ²
	Protagon solution.	Volume of diluent.	Total volume of diluted solution.	Recovered at -3° C. ¹	In filtrate at -3° C.	
	c.c.	c.c.	c.c.	Grams.	Gram.	%
1	200	800	1000	1.8	0.7	1.30
2	200	300	500	1.9	0.6	1.31
3	200	50	250	1.7	0.8	1.20
4 (control)	200	0	200	1.8	0.7	1.13

Precipitation was most rapid at room temperature in 4 (control) and slowest in the largest volume. Two hours after the mixtures were made up, precipitation had occurred in solutions 2, 3, and 4. Not even the faintest turbidity appeared in 1, however, until about 5 hours after the solution had been prepared, and another hour passed before flocks separated. Long before that time the temperature of the solution had dropped to that of 2, in which precipitation occurred early. Precipitates 1 and 2 were relatively light and bulky, whereas those in 3 and 4 were relatively compact and heavy.

The above quantitative data show that protagon cannot be completely precipitated from its solution by cooling to 0° C., an observation in harmony with facts tabulated on pages 96 and 109. The differences in the volumes used did not appreciably alter the amount of protagon that was recovered by the cooling process. The variations of the figures for content of phosphorus are hardly striking enough to justify any very definite conclusion on the question whether protagon is a mixture, and it does not seem probable that variations of volume of solvent in the fractionation experiments described on page 94 could have any important bearing on our results or conclusions. That larger proportions of diluent would emphasize the slight differences noted stands to reason.

Our results strongly suggest that phosphorus-free substance

¹ The amount of protagon that was dissolved in each sample of the filtrate (200 c.c.) was 2.5 grams.

² The phosphorus-content of the original protagon was 1.24 per cent. The estimated percentage content of phosphorus in the solid matter of the filtrates was (1) 1.1, (2) 1.0, (3) 1.3, (4) control, 1.5.

(or substance containing less phosphorus than protagonist) which was retained dissolved in the largest volume of refrigerated solution, was precipitated from the smallest volume of solution (control) under similar circumstances.¹ If these observations should be supported by additional experiments, we believe that the phrenosin contained in the protagonist will be found to behave in the manner indicated. If excess of the *solvent* used to purify protagonist by recrystallization should be avoided, in order to diminish the proportion of redissolved phrenosin (pseudocerebrin), it would seem natural to expect that the more concentrated the protagonist *solutions* would be in such comparative tests as those made in the last experiment, the larger would be the *proportion* of phrenosin in the precipitated protagonist and the lower the percentage of phosphorus in the latter. Our results accord with this view, and with the fact that, on reprecipitating protagonist, the substance in the alcoholic filtrate always has a higher phosphorus-content than the precipitated protagonist, an indication that the solubility of the phosphorus-free constituent or constituents of protagonist decreases more decidedly as the temperature falls than the solubility of those containing phosphorus.

VIII.—FRACTIONATION EXPERIMENTS. (I) APPLICATION TO
CRAMER'S PROTAGON OF THE FRACTIONATION METHOD
DESCRIBED BY LESEM AND GIES.

On page 76 are given the results of one of the experiments by Lesem and Gies which led them to conclude that protagonist was a mixture. The experiment referred to and the others of similar type reported by Lesem and Gies were carried out by Gies before Dr. Lesem was invited to assist him in completing their research.² We have applied their method to protagonist made by

¹ It is quite possible that, although the total weight of unprecipitated substance was practically the same in each solution, each mass was composed of different proportions of the same materials. With such a mixture as protagonist, the solids in the different filtrates might even have been different in kind, although this does not seem probable for the volumes of filtrates in the experiment referred to above.

² The results obtained by Gies were identical with a few unpublished observations of Chittenden and Frissell, and also with the results obtained by Thudichum prior to the work of all of these observers

the Cramer process, and, in order to prevent the possibility of unconscious duplication of any error that might have been made by Gies, the first three experiments described below, that repeated the method used by Lesem and Gies, were carried out by Dr. Posner without any other help from the writer than general suggestions. The results obtained by Dr. Posner with Cramer's protagon agree perfectly with the writer's previous results on protagon prepared by the Gamgee and Blankenhorn process, as the data recorded below show clearly.

A. FRACTIONATION OF PROTAGON 2 (SHEEP-BRAINS) PREPARED BY THE CRAMER PROCESS.—The results in this connection that were published by Lesem and Gies were obtained with protagon prepared from sheep-brains. We made this first experiment directly comparable to theirs by using a Cramer protagon (2) prepared from the same source. We proceeded as follows:

First protagon-product and the substance in its corresponding filtrate.—Exactly 20 grams of protagon 2, containing 1.07 per cent. of phosphorus, were treated with 1500 c.c. of 85 per cent. alcohol at 45° C. for nine hours. The mixture was repeatedly stirred. At the end of that time most of the substance remained undissolved. The mixture was filtered with all quantitative precautions. The protagon was separated from the extract by our customary refrigeration process (5 days). The filtrate from the protagon was evaporated to dryness on a water-bath.

Second protagon-product and the substance in its corresponding filtrate.—The undissolved portion of the original protagon was subjected to exactly the same treatment as the latter, except that extraction in the alcohol was continued for only seven hours. About half the original quantity of substance remained in solid form. Protagon and its corresponding filtrate were separated from this extract as before.

Third protagon-product and the substance in its corresponding filtrate.—The residue was treated as before, for ten hours. A considerable proportion of the protagon resisted solution. The filtered extract was separated into protagon and corresponding filtrate as previously.

Fourth protagon-product and the substance in its corresponding filtrate.—A final extraction of the residue was made under the same conditions as those that prevailed before, except that extraction was discontinued at the end of eight hours. The extract was separated into protagon and filtrate as before.

Insoluble portion of the original protagon.—About 15 per cent. of the original amount of protagon remained insoluble after the fourth treatment with warm alcohol.

Treatment of the products.—Each successive residual portion of protagon was washed with a moderate amount of 85 per cent. alcohol at 45° C.

Washing was conducted rapidly. The washings were added to the corresponding extracts before they were taken to the refrigeration room.

Each protagon-product that was obtained from the extracts by refrigeration (5 days at -3° C.) was washed first with a moderate amount of cold (0° C.) 85 per cent. alcohol, and then with a large excess of ether at the same temperature. The alcoholic and ether-washings of the freshly precipitated protagon-products were added to the corresponding filtrates from the latter and evaporated to dryness with each of them on a water-bath. The protagon-products were dried to constant weight at room temperature in desiccators over concentrated sulfuric acid. The solids from the corresponding filtrates were dried to constant weight in a few hours at 100° C. The loss of weight at the latter temperature was very slight, and there was no apparent decomposition (see p. 108).

Quantitative data in this connection are summarized below.

TABLE VIII.

FRACTIONAL PRODUCTS OBTAINED FROM CRAMER'S PROTAGON (SHEEP-BRAINS) BY THE METHOD OF LESEM AND GIES.

Fractional Product.	Weight in Grams.		Elementary Composition in %.		
	Separate.	Combined.	Phosphorus.	Nitrogen.	Sulfur.
I. Protagon fractions:	—	13.5	—	—	—
1. First product..	5.9	—	1.29	2.08	0.68
2. Second product	3.2	—	1.02	—	0.60
3. Third product..	2.3	—	1.00	1.90	—
4. Fourth product	2.1	—	0.68	—	0.54
II. Insoluble protagon (final residue)....	3.2	3.2	0.39	1.96	0.22
III. Solids in the filtrates from, and the washings of, the protagon products:	—	3.15	—	—	—
1. First product..	1.31	—	1.92	—	—
2. Second product	0.79	—	1.48	—	—
3. Third product..	0.56	—	1.28	—	—
4. Fourth product	0.49	—	1.02	—	—
Substance recovered....	19.85		—	—	—
Original protagon.....	20.00		1.07	2.07	0.62

B. FRACTIONATION OF PROTAGON (10) PREPARED FROM OX-BRAINS BY THE CRAMER PROCESS.—The preceding experiment was repeated with protagon made from the same source as Cramer's and by the Cramer method. Protagon 10 was used for the purpose, and 15.7 grams were taken. The phosphorus-content was 0.93 per cent. The volume of alcohol used for each extraction was 1000 c.c. The extractions continued for the same length of time in each case—eight hours. All other details of the preceding experiment were duplicated. Nitrogen and sulfur were not determined. Our quantitative data are given in Table IX.

TABLE IX.
FRACTIONAL PRODUCTS OBTAINED FROM CRAMER'S PROTAGON (OX-BRAINS)
BY THE METHOD OF LESEM AND GIES.

Fractional Product.	Weight in Grams.		Percentage of Phosphorus.
	Separate.	Combined.	
I. Protagon fractions:.....	—	8.0	—
1. First product.....	3.5	—	0.92
2. Second product.....	2.3	—	1.15
3. Third product.....	1.5	—	0.73
4. Fourth product.....	0.7	—	0.48
II. Insoluble protagon (final residue).	4.9	4.9	0.15
III. Solids in the filtrates from, and the washings of, the protagon products:.....	—	1.87	—
1. First product.....	0.80	—	2.24
2. Second product.....	0.43	—	1.90
3. Third product.....	0.44	—	1.82
4. Fourth product.....	0.20	—	0.80
Substance recovered.....	14.77		—
Original protagon.....	15.70		0.93

C. FRACTIONATION OF PROTAGON (20) PREPARED FROM SHEEP-BRAINS BY THE GAMGEE AND BLANKENHORN METHOD AND TREATED FURTHER BY THE CRAMER PROCESS.—The preceding experiment was repeated with protagon that had been prepared by the Gamgee and Blankenhorn method, and treated further by the Cramer process. Protagon 20 was used for the purpose; 15.7 grams were taken. Phosphorus-content was 1.55 per cent. Only two extractions were made—each time in 1000 c.c. of 85 per cent. alcohol at the usual temperature, for periods of five hours. The quantitative data are summarized below.

TABLE X.
FRACTIONAL PRODUCTS OBTAINED FROM GAMGEE AND BLANKENHORN'S PROTAGON (TREATED FURTHER BY CRAMER'S PROCESS) BY THE FRACTIONATION METHOD OF LESEM AND GIES.

Fractional Product.	Weight in Grams.		Percentage of Phosphorus.
	Separate.	Combined.	
I. Protagon fractions.....	—	13.00	—
1. First product.....	8.00	—	1.66
2. Second product.....	5.00	—	1.20
II. Insoluble protagon (final residue).	1.00	1.00	1.13
III. Solids in the filtrates from, and the washings of, the protagon products.....	—	1.20	—
1. First product.....	0.68	—	2.35
2. Second product.....	0.52	—	2.44
Substance recovered.....	15.20		—
Original protagon.....	15.70		1.55

General Observations.—Each of these experiments yielded results that accord perfectly with those obtained by Lesem and Gies. A new point is shown by the figures for nitrogen and sulfur in Table VIII. We have already expressed the belief that the insoluble portion of dried protagon consists largely of phrenosin (page 69), which contains neither phosphorus nor sulfur (page 74), but does contain about 2 per cent. of nitrogen. The agreement between this figure and that for nitrogen-content of the insoluble matter referred to (Table VIII), and also the greatly reduced content of sulfur in the insoluble portion (together with facts already noted), fortify our conclusion that the residue always obtained on "purifying" dried protagon (however frequently recrystallized) by resolution in alcohol, etc., consists chiefly of portions of phrenosin from the protagon-mixture.

As was stated above, the results of these experiments with Cramer's protagon are essentially the same as those obtained previously by Lesem and Gies with Gamgee and Blankenhorn's protagon (and by us since for the same make of protagon, page 101). They show beyond a doubt, we think, that Cramer's protagon was a mixture of essentially the same character as every other protagon hitherto prepared.

The following remarks by Lesem and Gies in reference to their own fractionation results apply here equally well, as an examination of Tables VIII–X will show:

"Among the points to be noted in the Tables (VIII–X) is the decreasing percentage content of phosphorus in each successive protagon and in the final insoluble residue. Also, the unusually high though diminishing proportion of phosphorus in the substances of the filtrates obtained each time protagon was separated at 0° C. Our method of fractional separation was that customarily employed in the *purification* of protagon. Here it was merely repeated more frequently than usual. Instead of obtaining purer protagons by the process, however, it appears that, with each successive precipitation, the substance itself changed in composition and, also, that variously composed products were liberated into the filtrates from the protagons at the same time. . . . These results were obtained by applying the usual purification process. They show, we think, that protagon is either a mixture of substances, or else a substance decomposing quite readily under the conditions of such treatments. If the latter conclusion appears to be more probable than the former, it must then be admitted that thus far no standard of purity for protagon has been

raised, which is not open to the objection that it is based on methods involving unavoidable decomposition." (Pp. 192-194.)

The only "decomposition" the writer then had in mind was that supposed, by many until recently, to be induced by warm alcohol—especially above 50° C. But the last alternative mentioned in the above quotation is a decidedly improbable one, as was shown, for example, in Gamgee's *latest* experiments¹ (*Text-Book*, 1880, pp. 429 and 440), in which, contrary to previous deductions, it was demonstrated that protagon resists for a long time the "decomposing" influence of even *boiling* alcohol.² Cramer prepared "pure" protagon from brain by extracting it with, and recrystallizing it from, *boiling* alcohol. Chemical decomposition is one thing, physical fractionation is another. The latter is accomplished by warm alcohol, the former apparently is not.

IX.—(2) FRACTIONATION OF PROTAGON BY REPRECIPITATION AT DIFFERENT TEMPERATURES.

The foregoing results naturally suggest numerous elaborate experiments that might demonstrate even more emphatically the fact that protagon is a mixture of substances. The following simple and very decisive experiments were suggested by the results described on page 88³:

¹ The earlier and opposite views expressed by Gamgee have been frequently quoted, but the amended conclusions stated in his *Text-Book* have not been noticed, it seems. (See page 70 of this paper.)

² "Pure protagon is remarkably rebellious to the action of even boiling alcohol, though that action be continued for hours" (Gamgee, *Text-Book*, 1880, p. 429). "Es verdient ferner hier noch erwähnt zu werden, dass wir bei der Darstellung sowohl, als auch beim Umkrystallisiren von Protagon die Lösungen nie über 45° C. erwärmten, da wir überzeugt sind, dass Liebreich mit Recht annimmt, dass sich dasselbe in alkoholischer Lösung über 50° C. erhitzt. zu zersetzen beginnt" (Gamgee and Blankenhorn, *Zeitschrift für physiologische Chemie*, 1879, iii, p. 280).

³ We were very agreeably surprised to find, shortly after the completion of this investigation, that Thudichum had previously obtained results, on *crude* protagon in similar experiments, with which our observations were in perfect accord. Thudichum's results in this connection have never been referred to by any other investigator, so far as we know. Consult Thudichum (1879 and 1886).

D. FRACTIONATION OF GAMGEE AND BLANKENHORN'S PROTAGON (18).—15.3 grams of protagon 18, containing 0.90 per cent. of phosphorus, were treated with 1500 c.c. of 85 per cent. alcohol at 45° C. for thirty hours. About one-fifth of the substance remained undissolved. At the end of the period indicated, the mixture was filtered quickly on apparatus kept at 45° C. in all its parts until the process was completed. The residue was washed with a moderate amount of 85 per cent. alcohol at 45° C. and the washings added to the filtrate. The residue weighed 3.2 grams.

Protagon-fraction obtained at 30° C.—The perfectly clear filtrate obtained at 45° C. was allowed to stand undisturbed at room temperature for forty-eight hours. A relatively bulky precipitate gradually collected. This precipitate consisted of long needles, arranged in irregular clusters and in large rosettes. Spheres were also seen. The mixture was filtered (30° C., July 7, 1905), the precipitate washed rapidly with a little 85 per cent. alcohol at room temperature and the washings added to the filtrate. The protagon-product weighed 6.8 grams.

Protagon-fraction obtained at -3° C.—The filtrate obtained at 30° C. was kept in the refrigeration room (-3° C.) twenty-four hours. A precipitate that was almost as bulky as the first gradually collected. It consisted of larger needles than those of the first product; they were in essentially the same arrangements. There were no spheres to be seen. The mixture was quickly filtered, rapidly washed with alcohol at -3° C. and the washings added to the filtrate as before. The product weighed 5.1 grams.

Solid matter in the filtrate from the second protagon-fraction.—The filtrate obtained at -3° C. was evaporated to dryness on a water-bath. The quantitative data of the experiment are summarized in Table XI (p. 101).

E. FRACTIONATION OF CRAMER'S PROTAGON (4).—The preceding experiment was repeated, with some variations, on a preparation of protagon made by the Cramer process, with essentially the same results as before.

Protagon-fraction obtained at 20° C.—10.3 grams of protagon 4, containing 0.95 per cent. of phosphorus, were rapidly and completely dissolved in 1500 c.c. of 85 per cent. alcohol at 65° C. The solution was kept at 50° C. overnight in a thermostat. In the morning the solution was only faintly turbid. During the day the temperature was allowed to fall gradually to 40° C. Only slight increase of the turbidity ensued. The solution was kept at room-temperature overnight. In the morning, when filtration was begun at a temperature of 20° C., a relatively bulky precipitate had collected. The product was washed as in the previous experiment at this point. 4.6 grams were obtained.

Protagon-fraction obtained at -3° C.—The filtrate obtained from the preceding product was kept in the refrigeration room 24 hours. Another relatively bulky precipitate was obtained and treated the same as the preceding product. Yield, 3.5 grams.

Solid matter in the filtrate from the second protagon-fraction.—The filtrate obtained at -3°C . was subjected to the action of a freezing mixture and kept at about -10°C . for six hours. A slight precipitate gradually collected, but it was not sufficient for quantitative determination of its phosphorus, and it was not filtered off. It speedily dissolved in the mother liquor as the temperature rose to that of the room. The clear solution was evaporated to dryness as usual. Yield: 19 grams. Our quantitative data are presented in Table XII (p. 102).

TABLE XI.

FRACTIONAL PRODUCTS OBTAINED FROM GAMGEE AND BLANKENHORN'S PROTAGON BY REPRECIPITATION AT DIFFERENT TEMPERATURES.

Fractional Product	Weight in Grams		Percentage of Phosphorus
	Separate	Combined	
I. Protagon fractions [*]	—	11.9	—
1. Product at 30°C . . .	6.8	—	0.72 ¹
2. Product at -3°C	5.1	—	1.20
II. Insoluble protagon	3.2	3.2	0.41
III. Solid matter in the filtrate from the product at -3°C	1.4	1.4	1.87
Substance recovered	16.5 ²		—
Original protagon	15.3		0.90

¹ This result might have been lower had the solution been less concentrated.

² By an oversight the largest portions of the products were not dried to constant weight. Portions of each fraction were used for the phosphorus-determinations and were removed from the main supply when further undetermined loss of weight had occurred. These portions were used for analysis and were dried to constant weight before treatment was begun. The weights recorded for imperfectly dried products are valuable only as showing approximately the proportions of the products separated. The next experiment was an improvement in this respect.

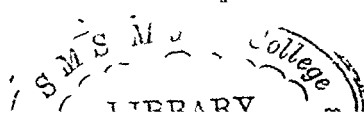


TABLE XII.

FRACTIONAL PRODUCTS OBTAINED FROM CRAMER'S PROTAGON BY REPRECIPITATION AT DIFFERENT TEMPERATURES.

Fractional Product. ¹	Weight in Grams.		Percentage of Phosphorus.
	Separate.	Combined.	
I. Protagon fractions.....	—	8.1	—
1. Product at 20° C.....	4.6	—	0.22
2. Product at -3° C.....	3.5	—	1.23
II. Solid matter in the filtrate from the product at -3° C.....	1.9	1.9	1.72
Substance recovered.....	10.0		—
Original protagon.....	10.3		0.95

General Observations.—The significance of the figures of the last two experiments (D and E) is so clear and the support the results give to the conclusion that protagon is a mixture of substances is so evident, that comment would be superfluous.

X.—(3) PROTAGON PREPARED BY THE GAMGEE AND BLANKENHORN METHOD, RECRYSTALLIZED TEN TIMES FROM 85 PER CENT. ALCOHOL, AND SUBJECTED TO FRACTIONATION.

As was stated on page 95, fractionation experiments A, B, and C were carried out by Dr. Posner unassisted by the writer in order that the method of Lesem and Gies (which had been carried out unassisted by Gies) might be applied independently to Cramer's protagon by another investigator, who would hardly repeat any mistakes that might have been made by Gies. Dr. Posner's results, as has been seen, agreed perfectly with those published by the writer three years ago. In spite, however, of

¹ In this experiment all of the protagon was purposely dissolved in the alcoholic solution prepared at the outset. The portion that ordinarily would be expected to persist as a residue (phrenosin), *i. e.*, on treatment with moderate quantities of 85 per cent. alcohol at 45° C., was dissolved in this experiment, but appeared for the most part in the precipitate obtained at room temperature, and which began to form above 50° C. (p. 94).

the decisiveness of the work described above, the writer concluded to put all of the preceding conclusions to the test of a special experiment, which was carried out by him without Dr. Posner's help. The experiment was planned, in the light of the accumulated data, to reveal, if possible, any facts that might fail to accord with any of the previous observations. Although Dr. Posner and the writer each took entirely independent shares of the work described in this paper, their results have been in perfect agreement.

The main plan of this experiment was to operate at the beginning with a very large quantity of protagon so that, in spite of the considerable loss that would occur at each step in the purification process, a fair amount of protagon might be available after the tenth recrystallization. This "repeated" recrystallization was carried far enough, doubtless, to meet every requirement of Gamgee's direction (page 68). It was expected to remove every trace of phrenosin and to yield the purest protagon ever made. The methods of fractionation that were used in the two preceding series were then applied jointly to the protagon that had been recrystallized ten times.

A. PREPARATION OF CRUDE PROTAGON.¹—500 sheep-brains were taken and protagon extracted by the Gamgee and Blankenhorn process. 36 liters of 85 per cent. alcohol were used for each extraction. An extract was obtained daily for ten days, at the end of which time extracts no longer became turbid on cooling. Each filtered extract was kept in the refrigeration room at -3° C. 24 to 48 hours. The precipitates in the first three extracts appeared to consist chiefly of cholesterin. All of the precipitates, as they were obtained, were put into a bottle containing about 5 liters of ether at room-temperature.² A large proportion of the combined crude products dissolved in the excess of ether. After the tenth precipitate had been dropped into the bottle, the ether-mixture was kept in the refrigeration room about 48 hours. It was then filtered rapidly at the low temperature, and the mass of crude protagon was washed roughly with cold ether.³

¹ This work was carried out mainly by our laboratory helper, Mr. Christian Seifert, under the writer's constant supervision.

² Thudichum's "buttery matter" was obtained in abundance from the alcoholic filtrates on evaporation nearly to dryness.

³ No effort was made to remove all of the cholesterin. Much of it remained.

B. RECRYSTALLIZATION AND PURIFICATION OF THE CRUDE PROTAGON.—The mixture of crude products was subjected, without previous drying,¹ to the process of recrystallization from solutions made at 45° C. in 85 per cent. alcohol. It resisted in marked degree the solvent action of the alcohol. Three daily extractions were made of it in about 12 liters of alcohol each time (total, 36 liters). The cream-colored precipitate softened and became doughy in the warm alcohol, and gradually, as it diminished, took on a decidedly yellow color. In spite of repeated stirring, about half of the material remained insoluble at the end of the treatment indicated. This residue was mainly cholesterol, and contained considerable phrenosin. The extract was removed by decantation. The extract was kept in the refrigeration room overnight and the protagon then filtered off. A sample was washed with ether and prepared for analysis, but the main mass without such washing was treated at once with 20 liters of alcohol² and extraction continued 24 hours. Again a considerable proportion of yellow matter remained insoluble. The quantity was much less, however, than after the previous extraction. This process of filtration, refrigeration, filtration, and resolution was repeated until a tenth recrystallization was effected. Further details in this connection are given in Table XIII.

Only about 35 grams of pure dry protagon (very bulky) were available at the end of the elaborate treatment described.

C. FRACTIONATION OF PROTAGON THAT HAD BEEN RECRYSTALLIZED TEN TIMES.—The previous fractionation methods (pp. 95 and 99) were applied jointly to this protagon, as follows:

Protagon-products 1 and 2 at 28° C. and -3° C., respectively, and the corresponding substance of the -3° C. filtrate.—16.2 grams of protagon, containing 0.93 per cent. of phosphorus, were treated with 1000 c.c. of 85 per cent alcohol at 40° C., 14 hours.³ Considerable residue remained which, when pressed with a stirring rod in the warm alcohol, was found to be soft and pasty in parts and powdery in others. The rod held such parts as were slightly melted and sticky, and thus exaggerated the effects, although the phenomenon noted might be expected to occur with a mixture like protagon.⁴ The mixture was filtered with all quantitative precautions.

¹ Its weight was not determined, but it was equivalent to about 2 kilos of dry matter.

² The alcohol was reduced in volume to prevent unnecessary losses. Further decreases of volume on resolution were made for the same purpose.

³ A trial test showed that the material was not more soluble than other samples, although in the recrystallization process it had dissolved very readily. In the latter case it was doubtless in a hydrated state and on that account more soluble.

⁴ We have repeatedly noted that uniformly powdered dry protagon melted in spots when alcohol at 45° C. was poured on it.

The warm filtrate from the residue was allowed to stand at room temperature (28°C.) 24 hours. There formed rapidly a relatively bulky, though light, precipitate consisting of beautiful rosettes of needles, a few *nodular masses*, and some *spheres*.¹ The precipitate was washed with a little 85 per cent. alcohol at the temperature of precipitation. The washings were added to the filtrate, and the precipitate transferred to 250 c.c. of ether at room temperature (28°C.), where it was allowed to remain 24 hours, when it was filtered off and rapidly washed on the filter paper with ether at room temperature. The ether-washings were saved.

The alcoholic filtrate obtained at room temperature was kept in the refrigeration room at -3°C. , 48 hours. This precipitate was microscopically the same as the first, excepting that it contained neither spheres, nor nodules and was washed first with alcohol and then with ether as in the case of the former precipitate. The ether-washings were combined with those of the previous precipitate. The alcoholic filtrate from the second precipitate was evaporated to dryness on a water-bath.

Protagon-products 3 and 4 at 28°C. and -3°C. and the corresponding substance of the -3°C. filtrate.—The insoluble protagon that remained after the first treatment with alcohol was extracted a second time under exactly similar conditions in 650 c.c.² of 85 per cent. alcohol, 27 hours. Protagon-products (3 and 4) of the same crystalline appearance as the two previous products were successively obtained at the same temperatures as the previous corresponding fractions. Further treatment of the protagon-products and the filtrate was the same as before. The ether-washings were combined with those of the first two protagon-products.

Protagon-products 5 and 6 at 28°C. and -3°C. and the corresponding substance of the -3°C. filtrate.—The insoluble matter remaining after the two previous extractions was still quite abundant. It was again extracted with 350 c.c. of 85 per cent. alcohol, 20 hours, under the conditions previously prevailing. The various products were obtained as before, and in microscopic appearance corresponded with those previously obtained (1-4).³ The ether-washings were combined with those from products 1-4.

¹ This observation is noteworthy because the protagon that we used consisted, on its tenth recrystallization, solely of rosettes of needles and loose needles and their entanglements. The solution from which the nodular masses and spheres were precipitated was doubtless sufficiently concentrated to cause the effects noted, and precipitation was also probably rapid enough, at the start at least, to favor them.

² Volume was reduced to keep the concentration about the same as before.

³ It was noticeable that spheres and nodules appeared only in the precipitates obtained at 28°C. This was probably due to the more rapid fall of the temperature to that point, than afterward in the refrigeration room.

TABLE XIII.

PRODUCTS OBTAINED ON RECRYSTALLIZING PROTAGON TEN TIMES.

No. of Reprecipitation.	Vol. of Alcohol for Resolution.	Protagon-Product.		Filtrate from Protagon: P. %	Insoluble Matter	
		Qualities.	P. %		Qualities.	P. %
—	Liters. 36	Crude product. Cream colored: cholesterin plates, spheres, needles, and amorphous matter.....	—	—	—	—
1	20	White: ² spheres, cholesterin plates, no needles.....	1.22	—	Yellow: cholesterin plates, spheres, needles, and amorphous matter ¹	—
2	16	Spheres very conspicuous, rosettes, isolated needles, cholesterin plates.....	—	—	Less yellow: same microscopic appearance and greatly reduced in amount...	—
3	14	Few spheres, some prismatic forms, long curved needles—entangled and separate; also rosettes and cholesterin plates.....	—	—	Nearly white: spheres and nodules. Small amount.....	0.78

¹ All of the filtrates from the protagons were evaporated to small volumes on a water-bath, after preliminary distillation of the bulk of the alcohol contained in them. Each contained white matter, which was similar to starch after being heated in water. The first four filtrates contained *much* cholesterin; the last four contained little of it. The figures for phosphorus-contents are given for substance which was not washed with ether. The following amounts of dry substance were obtained from the filtrates: 4-32.5 gr.; 5-14.5 gr.; 6-11.9 gr.; 7-10 gr.; 8-5.5 gr.; 9-4 gr.; 10-2.6 gr.

² All the subsequent protagonist-products were beautifully snow-white.

³ The residues rapidly diminished in quantity. The first amounted to about half the original crude product. The second was equal to about one-fifteenth the original mass.

TABLE XIII.—*Continued.*

PRODUCTS OBTAINED ON RECRYSTALLIZING PROTAGON TEN TIMES.

No. of Reprecipitation.	Vol. of Alcohol for Resolution.	Protagon-Product.		Filtrate from Protagon; P. %	Insoluble Matter.	
		Qualities.	P. %		Qualities.	P. %
4	12 Liters.	No spheres, few prismatic forms, few cholesterin plates, almost wholly rosettes, curved and bent needles.....	1.21	—	Trifling quantity, white: spheres and amorphous particles	0.80
5	10	Same as 4, but no prismatic forms and cauliflower forms in place of many rosettes	—	—	Hardly any residue: white. Spheres....	—
6	10	Same as 5, but no cholesterin plates ¹	—	1.42	None ²	—
7	8	No spheres but needle entanglements, rosettes and cauliflower forms.....	—	—	"	—
8	6	Same as 7.....	—	1.05	"	—
9	6	Chiefly rosettes.....	—	0.96	"	—
10	—	Almost wholly rosettes; short straight needles, rest needle entanglements.....	0.93	1.02	"	—

¹ Products 6–10 settled very slowly and were relatively bulky, phenomena evidently due to the relative increase of rosette forms.

² Protagon 6–10 dissolved very quickly and completely when put into alcohol warmed to 45°, and their solutions, like those previously obtained, were colorless and water-clear. Dry protagon dissolves only slowly even in excess of warm alcohol.

³ The analyzed protagon-samples were purified and dried as usual. Only necessary quantities were removed for this purpose. So much depended on the exactness of the results for phosphorus-content of the protagon, especially of the final product, that our colleague, Mr. W. N. Berg, was invited to check the results. Mr. Berg was told nothing about the characters of the products. His results agreed perfectly with the writer's. The results recorded above for proportions of phosphorus in the protagons are those obtained by Mr. Berg. We are indebted to him for his cordial coöperation.

Insoluble portion (residue).—The final residue when dry was hard and chalky. It consisted of granular matter, fragments of needles, and broken spheres. The latter doubtless represented the insoluble parts of the soft and pasty portions of the original protagonist, as noted above, and were probably formed from some of the needles by partial or complete melting of the latter. Application of Wörner and Thierfelder's method of effecting, in 85 per cent. alcohol at 50° C., crystallographic transformation of protagonist into phrenosin (cerebron) and other products, resulted positively.¹ The moist residue² was washed with ether under the same conditions as the protagonist-products and the washings were added to those of the latter.

Alcoholic filtrates.—The three alcoholic filtrates obtained at -3° C. were severally evaporated to dryness on a water-bath. This process was discontinued when the last portion of visible moisture disappeared. The residues were dried to constant weight in desiccators over concentrated sulfuric acid, instead of being subjected to a higher temperature as in previous experiments.

Ether-washings.—The ether-washings of all the precipitated protagonist-products and the residue were combined, as was said above. The temperature of the ether during the washing periods was in all cases about 28° C. We used ether at that temperature, instead of the *cold* ether usually employed, in order to favor, by change of method, any new fractionation that could have no tendency to effect chemical decomposition. By using ether at the higher temperature we favored removal from protagonist of matter that might have been insoluble in cold ether and thus have been retained in the protagonist. Our results bore out this idea. The combined ether-washings were evaporated and prepared for analysis under the same conditions as the alcoholic filtrates.

Quantitative data of this series of fractionations are given in Table XIV (page 109).

It will be observed that the data summarized in Table XIV verify in every particular the results obtained in the previous experiments, and prove beyond a doubt that protagonist of the very purest kind is not a chemical entity, but a mixture of chemical units.

¹ Certain other such protagonist-residues that were obtained in the previous experiments were found to exhibit the "umlagerung" described by Wörner and Thierfelder.

² Reasons for the occurrence of this residue were mentioned in the second footnote on p. 107. See also remarks on pages 68 and 69.

TABLE XIV.

FRACTIONATION PRODUCTS OBTAINED AT VARIOUS TEMPERATURES
FROM PROTAGON RECRYSTALLIZED TEN TIMES.

Fractional Product.	Weight in Grams.		Percentage of Phosphorus.
	Separate.	Combined.	
I. Protagon-fractions:	28°C.	0°C.	—
A—1. Product at 28° C.....	2.05	—	0.95
2. " " 0° C.....	—	1.69	1.22
B—3. " " 28° C.....	0.97	—	0.89
4. " " 0° C.....	—	1.12	1.12
C—5. " " 28° C.....	0.49	—	0.74
6. " " 0° C.....	—	9.59	6.91 ¹
6. " " 0° C.....	—	5.39	0.74
II. Insoluble portion (residue).....	—	—	—
III. Solid matter in the corresponding filtrates from the protagon products at 0° C:.....	—	—	—
A.....	—	0.60	—
B.....	—	0.31	1.68
C.....	—	0.28	1.19
IV. Combined ether-washings of I and II.....	—	—	2.23
Substance recovered.....	—	15.72	—
Substance taken.....	—	16.20	0.93

XI.—GENERAL OBSERVATIONS.

We consider that the results of these experiments necessitate abandonment of the idea that protagon is anything definite physically or chemically. We believe that the protagon hypothesis has interfered with the progress of neurochemical research. We hope our results will arouse a greater active interest in the chemistry and physiology of nerve constituents.

The new proteid, paranucleo-protagon, that was described by Ulpiani and Lelli (1902) as a definite substance, must be regarded as a mixture of products. It is our intention to begin at once an inquiry into this matter.

Our profitable study of Thudichum's neurochemical investigations; the agreement of practically all our results with his; and the evident disinclination, in various quarters, to take his work

¹ The total weights of protagon-products at the two temperatures were: 28°—3.51 grams; 0° C.—3.40 grams.

² The residues B and C were combined for analysis because of the small quantity of each.

seriously, impel us to express the conviction that the results which Thudichum obtained and the facts he collected in this connection (however unfortunate he was in his expressions regarding them) will be highly esteemed long after his personal allusions will have been generally overlooked.

XII.—SUMMARY OF GENERAL CONCLUSIONS.

Protagon is a mixture of substances. The characters of the constituents have not yet been determined, but it is evident that a phosphorus-free substance or substances are associated with one or more substances containing relatively large proportions of phosphorus.

Slight variations of the classical methods of preparation resulted in raising considerably the phosphorus-content of some of the products.

By fractionation at the same or different temperatures in 85 per cent. alcohol under conditions that could not effect chemical decomposition, protagon was partitioned into products very dissimilar in phosphorus- and sulfur-contents. These results confirm those of Thudichum and of Lesem and Gies.

Phrenosin, pseudocerebrin, and cerebron appear to have been identical products. The authors favor the retention of the name phrenosin for the substance, as proposed originally by Thudichum, its discoverer, instead of pseudocerebrin, the name selected by Gamgee, and instead of cerebron, suggested lately by Thierfelder in preference to pseudocerebrin. The authors have also proposed the name phrenosinic acid for the decomposition product called neurostearic acid by Thudichum and cerebronic acid by Thierfelder.

Phrenosin is probably invariably present in protagon.

By the use of Cramer's method of preparation, typical protagon was made, which could be fractioned without chemical decomposition into products of variable phosphorus-content similar to those obtained from protagon prepared by other methods. Cramer's method offers no particular advantages over previous processes of preparation. The authors believe that the method tends to increase the percentage of phrenosin in the crude product and that it therefore favors an increased percentage of phrenosin in the "purified" protagon.

The anomalous protagon obtained by Cramer ("homoprotagon," as he proposed to call it) is regarded by the authors as another addition to the evidence that protagon is indefinite chemically.

Protagon prepared by the Gamgee and Blankenhorn method and subsequently treated by Cramer's process appeared to be unaffected.

Long-continued extraction of "pure" dry protagon with ether in a Soxhlet apparatus failed to affect its composition. "Pure" *freshly precipitated* (hydrated) protagon yielded, to ether at room temperature, substance containing less phosphorus than the original protagon.

Results were obtained which suggest that the more dilute a solution of pure protagon may be in 85 per cent. alcohol, the higher the percentage of phosphorus tends to go in the product obtained by refrigeration.

Paranucleo-protagon is no more definite in chemical qualities than protagon itself.

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CERTAIN ASPECTS OF EXPERIMENTAL DIABETES.

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The problems connected with abnormal carbohydrate metabolism have been attacked from various experimental standpoints. Especially has attention been devoted to a study of the mechanism involved in the production of artificial diabetes in animals with a view to an explanation of the phenomenon of diabetes in man. Since the time of Claude Bernard it has been recognized that puncture of the floor of the fourth ventricle of the rabbit is followed by a transitory form of diabetes—the so-called *piqûre* diabetes. Equally well known are the experiments of Minkowski showing that removal of the pancreas leads to the production of a diabetic condition, persisting until the death of the animal. If, however, a portion of the pancreas is left intact no abnormal phenomena connected with carbohydrate metabolism are manifested. It is thus evident that at least two types of artificial diabetes may exist, the one being probably of nervous origin, the other in some manner more intimately dependent upon the function of the pancreas.

In the literature of physiology are to be found many records dealing with a third class of experimental diabetes—that provoked by certain chemical compounds. Thus, it is well known that phlorhizin¹ and uranium² salts are capable of calling forth a severe glycosuria which has been referred to the detrimental action of these substances upon the kidney cells.

¹ Cremer, *Ergebnisse der Physiologie*, 1902, I, Abtheilung 1, p. 803.

² Chittenden, *Transactions Connecticut Academy of Arts and Sciences*, 1888, viii, p. 1; also, *Studies in Physiological Chemistry from the Sheffield Laboratory of Physiological Chemistry, Yale University*, 1887-88, iii, p. 1; Lépine, *Comptes rendus de la société de biologie*, 1903, lv, p. 1289.

Coincident with this glycosuria there is a diminished sugar content of the blood. Various other substances, as adrenalin,¹ carbon monoxide,² ether,³ chloroform,⁴ morphine,⁵ curare,⁶ veratria,⁷ pyrogallol,⁸ salicylates,⁹ pilocarpine,¹⁰ amyl nitrite,¹¹ and potassium cyanide¹² possess the property of inducing a glycosuria, and as far as has been investigated, an accompanying hyperglycæmia has been noted.¹³ Obviously this type conforms more closely to the true diabetes, and the present investigation has accordingly been directed more particularly to it.

TECHNIQUE OF EXPERIMENTS.

The full-grown dogs employed had been fed for at least two days upon a meat diet and had received the last meal from fifteen to twenty hours previous to the experiment unless otherwise specified. During the trials the animals were kept under slight ether anæsthesia. Special attention was given the determination of the sugar content of the blood, the extent of glycosuria being incidentally noted. The sugar determinations were made according to the satisfactory¹⁴ method recommended by Vosburgh and Richards.¹⁵

¹ Blum, *Archiv für die gesammte Physiologie*, 1902, xc, p. 617; Herter and Wakeman, *Virchow's Archiv*, 1902, clxix, p. 479; Herter and Wakeman, *American Journal of the Medical Sciences*, 1903, cxxv, p. 46.

² Araki, *Zeitschrift für physiologische Chemie*, 1891, xv, p. 351.

³ Hawk, *American Journal of Physiology*, 1903-04, x, p. xxxvii, Seelig, *Zentralblatt für innere Medizin*, 1903, xxiv, p. 202

⁴ Kunkel, *Handbuch der Toxikologie*, 1901, p. 447.

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⁶ Araki, *ibid.*, p. 358.

⁷ Araki, *ibid.*, 1892, xvi, p. 458.

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⁹ Herter, *loc. cit.*

¹⁰ Doyon, Kareff, and Fenestrier, *Comptes rendus de la société de biologie*, 1904, lvi, p. 1.

¹¹ Araki, *Zeitschrift für physiologische Chemie*, 1891, xv, p. 546.

¹² Herter, *loc. cit.*

¹³ Hammarsten, *Text-Book of Physiological Chemistry*, 1904, p. 255.

¹⁴ By this method duplicate analyses of two samples of blood drawn from an artery at one time will give results differing from each other by 0.04 per cent. dextrose.

¹⁵ Vosburgh and Richards, *American Journal of Physiology*, 1903, ix, p. 35.

PIPERIDIN DIABETES.—DOES PIPERIDIN PRODUCE HYPERGLYCÆMIA?

Herter¹ has shown that when piperidin is painted upon the pancreas of the dog or injected intraperitoneally glycosuria is readily produced as a rule. At times the sugar elimination is very marked and may persist for several hours. Again, the glycosuria is very slight or may be entirely lacking, even though large doses of the drug have been administered. At the suggestion of Professor Herter this substance has been selected for more detailed study as a typical representative of the large number of drugs causing glycosuria.

Before attempting to determine the action of piperidin upon the blood sugar content, it seemed desirable to carry out control experiments to ascertain the effect of the slight anæsthesia employed in the investigation. The results of two such experiments are given in the following table:

TABLE I.
THE INFLUENCE OF ETHER ANÆSTHESIA ON THE SUGAR CONTENT OF THE BLOOD.

	Time.	Blood.	
		Amount Collected. Grams.	Sugar Content. Per Cent.
Experiment 1.	9.20	Anæsthesia complete.	Dog of 16.7 kilos.
	9.42	23.0	0.16
	10.26	21.0	0.18
	11.25	20.0	0.20
	12.40	30.0	0.18
Experiment 2.	9.50	Anæsthesia complete.	Dog of 16.9 kilos.
	10.04	22.7	0.16
	11.04	21.9	0.17
	12.36	23.0	0.18
	1.04	21.8	0.18
In Experiment 1 no urine was found in the bladder.			
In Experiment 2 the urine contained no reducing substance.			

From these results it is evident that ether anæsthesia in the degree employed in the above experiments does not induce any notable change in the sugar content of the blood or an appearance

¹ Herter, *loc. cit.*

of any reducing substance in the urine, even though the period of anæsthesia is relatively prolonged.

In the cases of piperidin glycosuria studied by Herter two attempts were made to determine whether it was accompanied by hyperglycæmia. In neither of the experiments was the sugar content of the blood changed, and the suggestion was made that a renal factor is associated with this form of glycosuria. These experiments have been repeated by the writer, and the influence of piperidin, applied to the pancreas, will be found in the appended table:

TABLE II.

THE INFLUENCE OF PIPERIDIN ON THE SUGAR CONTENT OF THE BLOOD.

	Time.	Blood.	
		Amount Collected. Grams.	Sugar Content. Per Cent.
Experiment 7.	9.25	Anæsthesia complete. Dog of 7 kilos.	
	9.37	19.0	0.13
	9.55	Pancreas painted with 5 c.c. of 2 per cent. solution of piperidin.	
	10.02	20.9	0.19
	10.20	19.1	0.15
	10.56	18.1	0.17
	11.56	16.3	0.19
	12.56 *	12.6	0.24
Experiment 8.	8.20	Anæsthesia complete. Dog of 8.5 kilos.	
	8.33	9.8	0.17
	8.45	Pancreas painted with 5 c.c. of 5 per cent. solution of piperidin.	
	9.03	10.0	0.18
	9.46	10.6	0.26
	10.46 †	9.8	0.32
Experiment 9.	9.05	Anæsthesia complete. Dog of 12.5 kilos.	
	9.18	13.4	0.24
	9.25	Pancreas painted with 5 c.c. of 4 per cent. solution of piperidin.	
	10.00	10.4	0.27
	10.31	10.3	0.32
	11.31	9.9	0.42
	12.31	10.0	0.31
	1.31 ‡	10.3	0.29

* Urine contained no sugar.

† Urine contained no sugar. Animal died of respiratory failure at 10.55.

‡ Urine contained a large quantity of sugar.

It appears, contrary to Herter's results, that piperidin may produce a significant hyperglycæmia coincident with glycosuria. The discrepancies between our results may be accounted for by the fact that the hyperglycæmia does not make its appearance at once, but is most in evidence one or two or even three hours after the application of the drug.

In general aspects piperidin diabetes resembles that caused by adrenalin. The extent of this resemblance was ascertained by determining whether the hyperglycæmia provoked by piperidin is also associated with an increased clotting time of the blood as has been demonstrated for adrenalin by Vosburgh and Richards.¹ No constant relationship between the sugar content of the blood and its clotting time could, however, be shown for piperidin.

IS THE HYPERGLYCÆMIA PROVOKED BY PIPERIDIN CAUSED BY AN
IRRITANT ACTION UPON, OR AN "INSULT" TO,
THE PANCREAS?

It is conceivable that increased sugar content of the blood may arise simply as a result of changes in the pancreas associated with an irritation, or "insult" (Flexner), purely mechanical in nature, as, for example, the effects of the operative procedure. Likewise of importance are the influences depending upon vascular changes in the gland. These changes must be considered in view of the fact that piperidin has a distinct influence upon blood pressure besides possessing very intense irritant properties. Pyrrol likewise is a very irritant substance possessing a marked action on blood pressure.

In the present experiments pyrrol was painted upon the pancreas to determine the influence of mere irritation of that organ with respect to changes in the sugar content of the blood. As a severe form of "insult," the gland was frozen with a spray of ethyl chloride; and to imitate mechanical injury due to operative procedure a solution of sodium chloride was painted upon the pancreas and the latter intentionally treated roughly.

¹ Vosburgh and Richards, *loc. cit.*

Examples and the results are given in the following table:

TABLE III.

THE INFLUENCE OF PYRROL, ETHYL CHLORIDE, AND SODIUM CHLORIDE ON CHANGES IN THE SUGAR CONTENT OF THE BLOOD.

	Time.	Blood.	
		Amount Collected, Grams.	Sugar Content, Per Cent.
Experiment 12.	2.50	Anæsthesia complete.	Dog of 18 kilos.
	3.09	21.6	0.08
	3.21	Ethyl chloride sprayed upon pancreas for 15 minutes. Gland frozen.	
	3.31	20.5	0.11
	3.55	20.9	0.08
	4.27	21.8	0.13
	5.23	24.2	0.09
Experiment 36.	8.50	Anæsthesia complete.	Dog of 12.5 kilos.
	9.00	16.1	0.17
	9.10	Pancreas painted with 7 c.c. of 4 per cent. solution of pyrrol. Gland much congested.	
	9.40	15.7	0.16
	10.15	18.5	0.15
	11.15	20.6	0.15
	12.15 *	14.3	0.17
Experiment 11.	2.05	Anæsthesia complete.	Dog of 18.0 kilos.
	2.20	22.0	0.16
	2.37	Pancreas painted with 0.9 per cent. sodium chloride solution.	
	2.50	22.0	0.19
	3.45	21.5	0.18
	5.10 *	20.4	0.15

* The urine contained no reducing substance.

According to the results detailed, the pancreas may be subjected to a considerable degree of irritation or "insult" without an appreciable change in the sugar content of the blood. The phenomenon of hyperglycæmia noted after applications of piperidin can not therefore be attributed to an irritant action or injury induced by the drug.

TO WHAT EXTENT IS THE PANCREAS THE EFFECTIVE FACTOR INVOLVED IN PIPERIDIN DIABETES?

Herter has laid particular emphasis upon the fact that adrenalin painted directly upon the pancreas, or given intraperiton-

eally, is far more effective in producing glycosuria than when introduced into the organism in other ways. This substance apparently exerts some specific action upon the cells of the pancreas, as a consequence of which sugar metabolism is temporarily deranged.

An endeavor has been made in the present investigation to determine whether piperidin likewise has a specific influence upon the pancreatic cells to which the phenomena observed may be attributed. Piperidin, injected intraperitoneally, resembles adrenalin in that the characteristic hyperglycemia and glycosuria are manifested. Here, however, the possibility of the drug coming in contact with the cells of the pancreas has not been excluded. The procedure of injecting piperidin intraperitoneally or of painting the substance upon the pancreas is far from ideal, since it involves the possibility of a direct local action of the introduced solution upon a number of contiguous organs in the peritoneal cavity. Furthermore, in view of the large absorbing surfaces to which the piperidin is thus exposed, various modes of intoxication at once suggest themselves. The writer has therefore made use of a method by which these objections are overcome. It consists in the application of the solution to the surface of the spleen after the removal of that viscus from its position in the peritoneal cavity through an opening in the abdominal wall. Throughout the experiment the organ is not replaced but is kept well packed with absorbent cotton moistened with warm isotonic salt solution. The packing is done in such a manner that the circulation is in no way impeded, and yet so that no portion of the solution can make its way into the abdominal cavity. With such an arrangement it is obvious that the effects noted after extraperitoneal applications of piperidin are not to be attributed to any direct action of the solution upon the pancreas or other organ in the abdominal cavity, and the conditions are such as to cause a *minimum* of injury to the abdominal viscera and peritoneum.

The employment of the method has permitted the demonstration of all the typical phenomena of piperidin diabetes without the introduction of any of the substance into the peritoneal cavity. It seems probable, therefore, that the influence of piperidin is exerted through the intervention of the circulation

rather than directly upon gland cells. This view is strengthened by the observation that the introduction of the piperidin, etc., directly into the circulation (intravenously) gives rise to diabetes.

Typical results are given in Table IV:

TABLE IV.

THE INFLUENCE OF PIPERIDIN ON THE SUGAR CONTENT OF THE BLOOD WHEN GIVEN INTRAPERITONEALLY, PAINTED ON THE SPLEEN, OR INJECTED INTRAVENOUSLY.

	Time.	Blood.	
		Amount Collected. Grams.	Sugar Content. Per Cent.
Experiment 6.	8.40	Anæsthesia complete. Dog of 8.5 kilos.	
	8.51	13.2	0.13
	9.00	Injected intraperitoneally 12 c.c. of 2 per cent. solution of piperidin.	
	9.30	14.4	0.17
	10.00	10.0	0.17
	11.00	11.6	0.36
	12.00	12.6	0.32
	1.00 *	13.2	0.26
Experiment 13.	10.05	Anæsthesia complete. Dog of 8 kilos.	
	10.13	11.6	0.16
	10.30	Spleen painted with 5 c.c. of 4 per cent. solution of piperidin.	
	10.44	11.6	0.17
	11.16	12.7	0.23
	11.44	12.4	0.24
	12.44	19.2	0.37
	1.05 *	10.9	0.38
Experiment 26.	10.40	Anæsthesia complete. Dog of 19 kilos.	
	10.47	14.9	0.06
	11.00	Injected slowly into the jugular vein 50 c.c. of 0.4 per cent. solution of piperidin.	
	11.50	15.1	0.19
	12.20	16.1	0.22
	1.20	16.6	0.20
	2.20 †	36.5	0.17

* Urine contained a large quantity of sugar.

† Urine contained no reducing substance.

In order to demonstrate further to what extent (if at all) the pancreas is the effective organ involved in this experimental glycosuria, observations were made on depancreatized animals for the purpose of ascertaining precisely the sequence of changes

in the sugar content of the blood incidental to the removal of the pancreas. The hyperglycæmia due to extirpation of the gland quickly culminates in a maximum blood sugar content within one hour after the operation, gradually falling to a constant high level which is maintained. The typical curve of the sugar content of the blood of normal animals does not reach its maximum until two to three hours after the introduction of the drug.

Applications of piperidin to the spleen in depancreatized dogs at a period when the sugar content of the blood has reached the stationary (high) level induce a subsequent increase in the percentage of sugar in the blood. Although the results of experiments of this nature can not be regarded as conclusive evidence, they indicate the possibility of a hyperglycæmia produced independently of the pancreatic mechanism.

In the appended Table V, a few typical experiments are given.

In view of the twofold source of sugar in (a) proteid and (b) glycogen within the body, the results of the above set of experiments are significant. They show that in extreme emaciation with greatly diminished store or complete absence of glycogen, the typical phenomenon of hyperglycæmia is no longer evoked by piperidin. A prolonged period of inanition, however, is necessary to lead to such an effect, in entire correspondence with Pflüger's well-known observations on the slow disappearance of glycogen from the dog in starvation. The results recorded above suggest that the increased sugar content of the blood after piperidin applications is dependent upon the glycogen content of the body.

IS THERE A RELATIONSHIP BETWEEN THE CHEMICAL STRUCTURE
OF CERTAIN DRUGS AND THEIR ABILITY TO PRODUCE
HYPERGLYCÆMIA?

The action of several substances closely related structurally to piperidin has been studied in an attempt to determine whether there is any definite relationship between the chemical structure and the ability of these compounds to produce hyperglycæmia. Thus, observations have been carried out with nicotine, coniine, pyridine, and pyrrole. Experiments have also been made with piperonal, a substance having no chemical relationship with the above-mentioned compounds.

TABLE V.

CHANGES IN THE SUGAR CONTENT OF THE BLOOD INCIDENT TO THE REMOVAL OF THE PANCREAS, FOLLOWED BY THE PAINTING OF PIPERIDIN ON THE SPLEEN.

Time.	Blood.	
	Amount Collected. Grams.	Sugar Content. Per Cent.
10.40	Anæsthesia complete. Dog of 14.7 kilos. Animal had fasted for 6 days.	
10.45	13.3	0.14
10.50-11.45	Pancreas removed. Wound sewed up.	
11.50	14.6	0.24
12.15	14.8	0.23
12.30	13.9	0.23
1.00	12.8	0.21
2.00	13.0	0.22
3.00	14.4	0.21
4.00	13.4	0.14
5.00 *	11.6	0.13
11.32	Anæsthesia complete. Dog of 13.5 kilos. Animal had fasted 6 days.	
11.43	12.0	0.15
11.45-12.25	Pancreas removed.	
12.29	13.2	0.20
12.34	Spleen painted with 5 c.c. of 4 per cent. solution of piperidin.	
1.00	13.0	0.23
1.15	11.3	0.24
1.45	13.4	0.29
2.45	13.5	0.32
3.45	11.3	0.33
5.40 †	12.3	0.27
10.15	Anæsthesia complete. Dog of 13.5 kilos. Animal poorly fed for 6 weeks. Emaciated.	
10.31	11.8	0.13
10.35-11.30	Pancreas removed.	
12.00	14.3	0.15
1.00	13.1	0.17
3.00	14.6	0.17
4.30	12.3	0.14
5.30	11.8	0.12
5.50	Spleen painted with 5 c.c. of 4 per cent. solution of piperidin.	
6.30	12.2	0.16
7.30	11.9	0.14
8.30 *	11.1	0.17

* The urine was free from reducing substances.

† The bladder was empty.

TABLE VI.

HYPERGLYCÆMIA AND COMPOUNDS OF CHEMICALLY RELATED STRUCTURE.

Time.	Blood.	
	Amount Collected. Grams.	Sugar Content. Per Cent.
Experiment 30.	11.27 Anæsthesia complete. Dog of 10 kilos.	
	11.35 18.0	0.12
	11.46 Pancreas painted with 0.5 c.c. of 1 per cent. nicotin solution.	
	11.53 15.8	0.19
	11.59 Pancreas painted with 1 c.c. of 1 per cent. nicotin solution.	
	12.18 13.2	0.16
	12.30 * 20.3	0.24
Experiment 34.	11.50 Anæsthesia complete. Dog of 10.8 kilos.	
	11.55 12.0	0.14
	12.10 Pancreas painted with 2 c.c. of 2 per cent. pyridin solution.	
	12.20 20.0	lost
	12.25 Pancreas painted with 2 c.c. of 2 per cent. pyridin solution.	
	12.45 17.4	0.19
	2.00 19.1	0.23
	2.50 16.2	0.18
Experiment 31.	3.20 * 16.9	0.23
	10.10 Anæsthesia complete. Dog of 11.3 kilos.	
	10.15 10.0	0.09
	10.28 Pancreas painted with 2 c.c. of 0.5 per cent. coniin solution.	
	10.34 17.0	0.15
	11.00 18.5	0.14
	11.03 Pancreas painted with 1 c.c. of 0.5 per cent. coniin solution.	
	11.18 25.0	0.16
Experiment 37.	11.32 19.0	0.19
	12.25 * 15.8	0.23
	8.30 Anæsthesia complete. Dog of 6 kilos.	
	8.43 14.2	0.14
	8.50 Pancreas painted with 5 c.c. of 2 per cent piperonal solution.	
	9.25 Pancreas painted with 5 c.c. of 4 per cent. piperonal solution.	
	9.50 11.0	0.18
	10.30 15.1	0.18
	11.15 19.0	0.19
	12.00 * 14.7	0.20

* The urine contained no reducing substance.

It will be observed from the results of the experiments given above that pyridin, nicotin, and coniin, when painted upon the pancreas, all possess the property of inducing a significant increase in the sugar content of the blood. Pyrrol, however, which is closely related to these substances in structure, is without influence (see Table III, p. 118). On the other hand, piperonal has no intimate relationship to these drugs and yet induces a slight hyperglycæmia. The existence in these cases of a definite relationship between chemical structure and ability to provoke hyperglycæmia can not therefore be shown.

A THEORY TO ACCOUNT FOR THE DIABETES CAUSED BY PIPERIDIN,
ETC.

In many of the experiments with piperidin and related compounds it was observed that disturbances (dyspnœa) of respiration arose, and indeed in some cases to such an extent that death frequently ensued as a result of respiratory failure. Such observations suggest a possible influence of disturbances of respiratory processes on the sugar content of the blood. To determine to what extent interference with respiration may account for the appearance of hyperglycæmia two methods have been employed, (a) a study of the sugar content of the blood as it is influenced by dyspnœa, and (b) a study of the behavior of oxygen inhalation on the effects of piperidin.

Two methods have been followed to produce dyspnœa. The first consists in regulating the gaseous exchange through a tracheal cannula. The second, a much more satisfactory method,¹ is briefly as follows: paraffin, to which enough olive oil has been added so that it solidifies a little above the temperature of the body, is injected through the carotis communis into the arteria vertebralis. The carotid of the opposite side is ligatured. Immediately after the injection of the melted paraffin the animal ceases to breathe. By this method the respiratory centre is excluded and the necessary artificial respiration may be varied at will through a tracheal cannula attached to an artificial respiration apparatus.

¹ Marckwald, *Zeitschrift für Biologie*, 1890, xxvi, p. 259.

TABLE VII.

THE INFLUENCE OF DYSPNŒA ON THE SUGAR CONTENT OF THE BLOOD.

	Time.	Blood.	
		Amount Collected. Grams.	Sugar Content. Per Cent.
Experiment 40.	8.33	Anæsthesia complete.	Dog of 12.5 kilos.
	8.40	20.16	0.16
	9.00	Dyspnœa produced at short intervals by tracheal cannula.	
	9.30	19.1	0.18
	10.15	17.1	0.19
	11.15	19.0	lost
	11.40 *	20.0	0.22
Experiment 41.	9.45	Anæsthesia complete.	Dog of 8.5 kilos.
	9.55	22.5	0.13
	10.13	Dyspnœa produced as above.	
	10.45	13.0	0.13
	11.13	16.8	0.17
	11.40	17.0	0.19
	12.15 *	21.0	0.21
Experiment 43.	9.45	Anæsthesia complete.	Dog of 10.5 kilos.
	10.15	20.4	0.11
	10.22	Injection of 1 c.c. paraffin. Artificial respiration. Dyspnœa induced at intervals. Blood never venous.	
	11.10	20.4	0.15
	11.45	21.5	0.19
	12.30 *	30.3	0.38
Experiment 44.	9.25	Anæsthesia complete.	Dog of 8.7 kilos.
	10.00	15.2	0.19
	10.22	Respiration stopped by paraffin injection.	
	10.27	21.00	0.19
	10.40	16.0	0.19
	11.30	38.0	0.42
	11.55 *	34.0	0.34

* The urine contained no sugar.

The results of these experiments indicate that suppression of the respiratory processes to the point of dyspnœa is productive of a decided increase in the sugar content of the blood. The fact that lack of oxygen may give rise to a very marked glycosuria

was long since pointed out by Araki,¹ and was later confirmed by Lépine and Boulud.² Dyspnoea caused by sudden seizures of epilepsy may also lead to sugar in the urine, as has been shown by Micha and Reynoso.³

It is well known that piperidin, coniin, nicotin,⁴ and pyridin⁵ exert a very powerful influence on the respiratory centre, leading to dyspnoea, and finally ending in respiratory failure. This fact suggested the possibility that these substances may evoke hyperglycæmia indirectly by acting upon the respiration and causing a diminished oxidation on the part of the organism. Assuming the probability of such an explanation it is reasonable to suppose that if the tissues could be well supplied with oxygen, and dyspnoea prevented, the hyperglycæmia resulting from the application of piperidin, etc., might be eliminated.

Experiments to test this supposition have afforded the typical results given in Table VIII. In order to administer sufficient oxygen a mask fitting over the nose of the animal was connected with a tank of oxygen.

Such data justify the conclusion that oxygen administration suffices to prevent the increase of the blood sugar content usual after applications of piperidin to the pancreas.

From the observations that dyspnoea may at times call forth hyperglycæmia and glycosuria, that piperidin readily produces dyspnoea and as a consequence hyperglycæmia and glycosuria; and that the latter conditions may be prevented by the administration of oxygen, the following theory to account for the mechanism of piperidin diabetes is indicated. Piperidin provokes experimental diabetes, not as a result of any specific action of the drug upon the organs governing the carbohydrate metabolism of the body, but indirectly as a consequence of its influence upon the respiratory centre. The resulting dyspnoea leads to defective oxidative power on the part of the organism with a consequent accumulation of sugar in the blood, the excess

¹ Araki, *Zeitschrift für physiologische Chemie*, 1891, xv, p. 335.

² Lépine and Boulud, *Comptes rendus de l'Académie des Sciences*, 1902, cxxxiv, p. 1341.

³ Micha and Reynoso, *ibid.*, 1853, xxxvi, p. 230.

⁴ Moore and Row, *Journal of Physiology*, 1897-98, xxii, p. 273.

⁵ Brunton and Tunnicliffe, *ibid.*, 1894, xvii, p. 272.

of which may or may not find its way into the urine. It is not at all likely that lack of oxygen *per se* in the tissues is the direct cause of the increase of sugar in the blood. A much more probable explanation of the phenomenon is that the oxidative enzymes, the oxidases, which are very sensitive to changes in oxygen in their environment, are influenced in such a manner as to be incapable of performing their normal function. It is also possible that there is a simultaneously increased transformation of glycogen into sugar in the liver as a result of the diminished supply of oxygen to that organ.

TABLE VIII.

THE INFLUENCE OF OXYGEN ADMINISTRATION ON PIPERIDIN DIABETES.

	Time.	Blood.	
		Amount Collected. Grams.	Sugar Content. Per Cent.
Experiment 39.	8.50	Anæsthesia complete. Dog of 13.7 kilos.	
	8.58	18.2	0.16
	9.00	Animal allowed to breathe oxygen. 32 liters used.	
	9.06	Pancreas painted with 5 c.c. of 4 per cent. piperidin solution.	
	9.42	34.6	0.15
	10.12	20.2	0.17
	11.12	19.8	0.16
	12.00	19.1	0.17
	12.15	Dyspnœa produced at intervals.	
	12.21	33.1	0.15
Experiment 38.	12.36	30.2	0.15
	12.59 *	24.2	0.24
	8.55	Anæsthesia complete. Dog of 17.0 kilos.	
	9.07	30.0	0.16
	9.10	Animal allowed to breathe oxygen. 40 gallons used.	
	9.12	Pancreas painted with 5 c.c. of 4 per cent. piperidin solution.	
	9.53	18.8	0.14
	10.18	24.2	0.13
	11.19	26.0	0.15
	11.48	20.2	0.15
	12.20 *	25.5	0.13

* The urine contained no reducing substances.

A glance at the tables constructed from typical examples of piperidin diabetes reveals many instances in which glycosuria is wanting. This is readily explained by the fact that small doses of the drug were given, and hence the increase in the sugar content of the blood was not sufficient to cause elimination by the kidneys. The absence of glycosuria in many of these experiments makes the theory outlined above even more tenable, for it has been shown that during asphyxia¹ itself there may be no glycosuria, and that the latter appears only after the return of normal respiration. The quantity of sugar in the urine may then reach six per cent. The glycosuria becomes maximal about three hours after the resumption of normal breathing and continues for six to eight hours.

In the work of Herter with adrenalin, piperidin, and other substances, *prominence was given to the fact that nearly all the drugs which cause glycosuria when painted upon the pancreas are reducing substances.* A relationship between reducing power and ability to provoke glycosuria was therefore suggested by Herter, but he also pointed out that the quantities of the drugs introduced were far too small to take sufficient oxygen from the tissues to cause any lack of it in specific groups of cells. Furthermore, such a view would not account for the observation that potassium cyanide, which has little or no reducing power, when painted upon the pancreas leads to the appearance of very significant quantities of sugar in the urine. The reason for the behavior of potassium cyanide at once becomes obvious when viewed in the light of the theory advanced by the present writer, for potassium cyanide has a very decided influence in evoking dyspnoea.²

Is the theory outlined above applicable to substances other than piperidin, pyridin, nicotin, and coniin? The pharmacological study of those compounds (mentioned in detail on page 114) which give rise to hyperglycæmia and glycosuria reveals at least one feature common to all, namely, an influence upon the respiratory centre bringing about death by failure of respiration. No experiments have been made with these compounds in the present investigation. Nevertheless, in view of their related

¹ Kunkel, *Handbuch der Toxikologie*, 1901, p. 348.

² Kunkel, *ibid.*, p. 503.

effects on respiration and their tendency to produce diabetes, it seems probable that the same theory which suffices to explain the diabetic mechanism in piperidin diabetes will admit of further application in the other cases under discussion.

The experiments in which it is shown that oxygen administration prevents the hyperglycæmia and glycosuria due to the action of piperidin are not isolated examples, for Penzoldt and Fleischner¹ and Sauer² have shown that after poisoning with curare no sugar is found in the urine if artificial respiration is maintained. Ether glycosuria can be prevented in a similar manner.³

THE EXPERIMENTAL GLYCOSURIA CAUSED BY ADRENALIN.

At first thought the glycosuria produced by adrenalin might be placed in the category with the substances discussed above, for it has been shown repeatedly that adrenalin has a specific⁴ action upon the respiratory centre and that in many instances death results from respiratory failure.⁵ Closer examination of the phenomena connected with adrenalin diabetes shows, however, that in one important respect the conditions are different from those that obtain in piperidin diabetes. In the former, hyperglycæmia and glycosuria occur very quickly following injections or applications of the drug, whereas with piperidin a considerable interval elapses previous to the manifestation of the phenomena. An experiment was tried to determine whether free access of oxygen would prevent the appearance of the symptoms usual after applications of adrenalin to the pancreas. The procedure employed involved the exclusion of the respiratory centre, followed by artificial respiration. A very marked hyperglycæmia and glycosuria resulted despite the favorable conditions. This observation suggests that the action of adrenalin is unique, and for the present it must be placed in a class quite distinct from the diabetes-producing compounds thus far investigated.

¹ Penzoldt and Fleischner, *Virchow's Archiv*, 1882, lxxxvii, p. 210.

² Sauer, *Archiv für die gesammte Physiologie*, 1891, xlix, p. 423.

³ Seelig, *Zentralblatt für innere Medizin*, 1903, xxiv, p. 202.

⁴ Neujean, *Archives internationales de Pharmacodynamie et de Thérapie*, 1904, xiii, p. 45.

⁵ Drummond, *Journal of Physiology*, 1904, xxi, p. 31.

SUMMARY.

When piperidin, nicotin, etc., are painted upon the pancreas of dogs, hyperglycæmia is a constant symptom. The application of the substances directly to the pancreatic tissue is not essential, since similar results may be obtained by painting the spleen, by intraperitoneal injection, or by direct introduction into the blood. It is probable, therefore, that the influence of these drugs is exerted through the intervention of the circulation rather than directly upon the gland cells to which the substances have been applied. The experimental diabetes is not due to an irritant action upon the pancreas, or an "insult" to it, for the hyperglycæmia may be induced independently of this gland. The experiments indicate that the action of piperidin, etc., is not specific, nor is the pharmacological action of the drugs necessarily related to their chemical structure.

The explanation offered for the hyperglycæmia and glycosuria provoked by such substances as piperidin, pyridin, coniin, nicotin, curare, ether, chloroform, morphine, carbon monoxide, pyrogallol, etc., does not assume any specific action (such as deprivation of oxygen) upon any particular gland like the pancreas. These substances have more or less effect upon the respiratory centre, producing dyspnœa. Dyspnœa, however induced, calls forth a marked hyperglycæmia and glycosuria without the intervention of any drugs. The typical rise in the sugar content of the blood produced by piperidin fails to appear when oxygen is administered. It is likely, therefore, that this experimental diabetes involves defective oxidation of carbohydrate material. Hyperglycæmia is the constant factor, and glycosuria follows whenever there is a sufficient accumulation of carbohydrate in the blood.

The considerations presented are inadequate to explain the phenomenon of adrenalin diabetes.

ON SULPHATE AND SULPHUR DETERMINATIONS.

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I.—THE CHEMICAL COMPOSITION OF BARIUM SULPHATE PRECIPITATES.

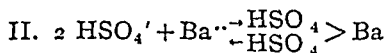
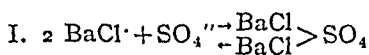
Up to a short time ago it was thought that the chemical reactions which take place when barium chloride is added to a sulphate solution are of the simplest kind, the products being BaSO_4 and MCl . The presence of "impurities" in barium sulphate precipitates had therefore to be explained as a physical phenomenon. It was said to be due to "occlusion."

Last year, however, Hulett and Duschak published a most interesting paper on the presence of chlorine in barium sulphate precipitates.¹ These investigators found that carefully washed

¹ Hulett and Duschak, *Zeitschrift für anorganische Chemie*, xl, p. 196, 1904.

and dried (140° C.) barium sulphate contains not only barium chloride, as had been previously found by Richards, but also some kind of chlorine combination which splits off hydrochloric acid at higher temperatures. I have several times repeated their experiments and obtained similar results. The remaining precipitate is not alkaline after the hydrochloric acid has been driven off. The latter is therefore not formed by decomposition of "occluded" barium chloride. A number of experiments cited by Hulett and Duschak show, in fact, very clearly that the hydrochloric acid formed can neither be due to "occluded" hydrochloric acid nor to the decomposition of barium chloride. The explanation of this curious phenomenon advanced by Hulett and Duschak explains at the same time in a most satisfactory manner why it is so difficult to secure pure barium sulphate precipitates.

The sulphuric acid solutions do not dissociate completely into H^+ and SO_4^{--} ions. Some HSO_4' ions remain in the solution. Similarly in the case of the barium chloride, the dissociation results in the formation of some $BaCl^+$ ions in addition to the Cl^+ and Ba^{++} ions. On the assumption of such a partial dissociation, $BaCl^+$ ions could combine with HSO_4' ions and give the salt $BaClHSO_4$, which on being sufficiently heated would split off HCl and leave neutral $BaSO_4$. This reaction would, however, not explain the presence of fixed chlorine and the excessive weight of the precipitates after ignition. But these two facts, as well as the loss of hydrochloric acid, would be perfectly explained if the following two equations can be accepted as representing a part of what does take place:



When the salt $\overset{HSO_4}{HSO_4} > Ba$ is heated alone, it should give off sulphuric acid; when heated together with more than the equivalent quantity of $\overset{BaCl}{BaCl} > SO_4$, hydrochloric acid should be formed; and when heated with less than the equivalent of $\overset{BaCl}{BaCl} > SO_4$, both

sulphuric and hydrochloric acids should be formed. Hulett and Duschak tried in vain to find that any traces of sulphuric acid are given off when barium sulphate precipitates are ignited. This is, however, scarcely to be wondered at. The fact that the ignited barium sulphate precipitates tend to weigh too much must be taken to mean, in the light of the above equations, that the salt, $\text{BaCl} > \text{SO}_4$, is formed in larger quantities than the second salt, $\text{HSO}_4 > \text{Ba}$. And since both are present only in comparatively small amounts the free sulphuric acid escaping in the presence of $\text{BaCl} > \text{SO}_4$ might easily be too minute for detection.

Hulett and Duschak advance the above equations only as a possible explanation. It is clear that the explanation needs the demonstration that barium sulphate precipitates can lose sulphuric as well as hydrochloric acid when ignited. Our hope of demonstrating this would probably be very slight if it were true that barium sulphate precipitates always tend to weigh too much. But this is fortunately not the case. I have found conditions which invariably give too low analytical results, notwithstanding the fact that the precipitation is very complete; and the barium sulphate obtained under such conditions does then indeed lose not only hydrochloric but also sulphuric acid when gently ignited.

The precipitates are obtained in the following manner: To a dilute potassium sulphate solution (100 to 200 milligrams per 100 c.c. H_2O) is added hydrochloric acid (about 3 c.c. of concentrated acid for each 100 c.c. of solution). A weak barium chloride solution (2.5–5.0 per cent.) is added drop by drop in the cold, while the solution in the flask is constantly shaken. The precipitation is complete in a few minutes, and the filtration and washing can be begun at once. The results obtained are the same whether the filtration is made at once or after twenty-four hours' standing. In neither case do the precipitates pass through the filter. The washed precipitates, dried in a platinum dish at about 140°C . and transferred to a hard glass tube, when heated give off very quickly a colorless liquid having the odor of hot sulphuric as well as that of hydrochloric acid. The glass

tube is cut between the precipitate and the liquid. The latter is rinsed out with water and filtered when necessary. The filtrate gives abundant precipitates with silver nitrate, and also with barium chloride.

The conditions described above are not the only ones which give complete precipitation yet too low results. Others representing more or less perfectly the conditions sometimes prevailing in actual analytical work will be considered in a later part of this paper (see Sections IV and V). The results stated constitute strong evidence in favor of the hypothetical reactions by means of which Hulett and Duschak explain the presence of both volatile and fixed chlorine in barium sulphate precipitates. But if those reactions occur there must be still others which come into play in barium sulphate precipitations. In the case of salts of sulphuric acid we should have not only SO_4'' ions and HSO_4' but also MSO_4' ions which can take part in the reaction. In such cases the precipitates may therefore also contain salts corresponding to the formula $\frac{\text{MSO}_4'}{\text{MSO}_4} > \text{Ba}$.

These salts, as well as a corresponding amount of $\frac{\text{BaCl}}{\text{BaCl}} > \text{SO}_4$, would be stable at moderate ignition temperatures, and the analytical results would inevitably be too high. This condition is strikingly illustrated when a neutral barium chloride solution is allowed to drop into a neutral potassium sulphate solution. The figures obtained are very much too high even when the precipitate before the filtration is digested for a long time with dilute hydrochloric acid (see p. 146).

From the above theoretical considerations it follows that correct barium sulphate values are to be obtained only by carefully adjusting the conditions until the precipitates given are found to possess the correct weight. It may be thought that this is self-evident, and that the correct conditions have already been worked out by a number of careful investigators. I venture to affirm, however, that while this may have been done for sulphuric acid solutions, it certainly has not been done for mixed sulphate solutions. The conditions described by Richards,¹ for example, would give very erroneous results in the

¹ Richards and Parker, *Proceedings of the American Academy*, xxxi, p. 71, 1895; *Zeitschrift für anorganische Chemie*, viii, p. 420, 1895.

presence of a single gram of potassium chloride. For very careful work Richards and also Hulett and Duschak determine the chlorine in the ignited precipitate and subtract an equivalent quantity of barium chloride from the ascertained weight. It seems to me, however, that this measure can scarcely be adequate except in the case of pure sulphuric acid solutions. In the case of sulphate solutions when the precipitate may contain the salt, $\frac{\text{MSO}_4}{\text{MSO}_4} > \text{Ba}$, such a correction might make the results more erroneous than they would be without it. The correction would certainly be uncalled for in case the precipitation is made in the presence of potassium chloride, because the uncorrected results are then already too low.

II.—THE PHYSICAL CONDITION OF BARIUM SULPHATE PRECIPITATES.

It seems to be an almost universal opinion that barium sulphate precipitates are very prone to take the shape of exceedingly minute amorphous particles which pass through all but the very best brands of filter paper which have been especially prepared to retain such precipitates. Numerous little chemical tricks designed to render the precipitates fit for the filter are consequently to be found in the literature. The same idea is met with in the elaborate methods that have been described for the preparation of crystalline barium sulphate. Yet it is certainly not difficult to secure in the course of ordinary analytical work barium sulphate precipitates which consist exclusively and at once of good-sized, well developed crystals. All that is necessary is to have both the sulphate and the barium chloride solution dilute, and to add the latter slowly (drop by drop). It is not even necessary that either solution should be hot; but the crystals obtained in the cold are not so uniform and are of a different kind from those obtained at higher temperatures. A cold two per cent. barium chloride solution can actually be "dumped" into a cold sulphate solution containing the equivalent of 100 milligrams of BaSO_4 per 100 c.c., and yield a precipitate which consists exclusively of large uniform bunches of rosette-like crystals.

These facts are not without importance in connection with sulphate determinations. In most laboratories it is customary to let sulphate precipitates digest in a hot place for several hours, or over night, in order to be sure of clear filtrates. I have repeatedly examined barium sulphate crystals at the end of a few minutes and at the end of twenty-four hours, and could observe no appreciable difference in the size or uniformity of the crystals. And the filtrates run through quite as clear in one case as in the other. In all ordinary sulphate determinations it is therefore perfectly safe to let the solution containing the precipitated barium sulphate cool at once after having added the barium chloride, and to filter as soon as it is cold. It would, however, be fatal to make this rule general. It will be shown in a later section (IX) that it is sometimes necessary to let the barium sulphate precipitates digest forty-eight hours before filtering. But in these cases the delay is not for the purpose of letting the precipitates "settle," nor for the purpose of securing clear filtrates, but only for the purpose of getting complete precipitations.

III.—THE PRECIPITATION OF BARIUM SULPHATE IN THE HEAT.

In order to secure precipitates which are crystalline and which will filter clear at any time, it is desirable, if not necessary, that both the sulphate and the barium chloride solutions should be dilute. The sulphate solution should not yield over 200 milligrams of barium sulphate per 100 c.c. Fresenius recommends a concentration equal to about 1 milligram of barium sulphate per c.c. of solution. The concentration and the rate of addition of the barium chloride, though frequently disregarded, are, however, even more important. Some text-books on physiological chemistry recommend saturated barium chloride solutions for the precipitation of sulphates in urine!

Ten per cent. barium chloride is the strongest concentration with which I have been able to obtain satisfactory figures. And this solution must not be added directly from the bottle, nor from a test tube, nor from a freely flowing pipette. It must be added at a rate not exceeding 5 c.c. per minute. Unless this

precaution is observed, the results will frequently be too high, and will always be uncertain.

Automatic droppers for the addition of barium chloride can be made in a few minutes from plain "calcium chloride tubes." The tubes are cut about 3 cm. above the bulb and about the same distance below the bulb. The small ends are then melted together until only capillary openings remain. They are especially useful when the precipitation is made in Erlenmeyer flasks, as is the case in this laboratory. A small dent should be made in the bulb by means of a flame. This allows the steam to escape when the bulb rests on the neck of the flask. The small end of the droppers can be bent so as to deliver the barium chloride solutions on the sides of the flask, but according to my experience nothing is gained by this device. Richards recommends adding the barium chloride on the sides, but he used very concentrated solutions of sulphuric acid in his work.

How much barium chloride should be used for each precipitation? In this laboratory we never use more than 10 c.c., and in most cases only 5 c.c. of 10 per cent. solution. The latter amount is sufficient to precipitate about half a gram of barium sulphate. Repeated comparisons have led me to the conviction that as accurate and certain results are to be obtained from 150 milligrams of barium sulphate as with two or three times that amount. This may seem doubtful to those accustomed to work with larger amounts; but in ordinarily careful work the errors in the results are not due to faulty measurements of the sulphate solutions, nor to the weighing of the precipitates, nor to their solubility. They are due to unsuitable conditions in the precipitation, and to losses during the ignition, and these sources of error are as disastrous to large as to small amounts of barium sulphate. A very slight excess of barium chloride is sufficient for the precipitation, if the other conditions are approximately suitable; and if they are not, the addition of more barium chloride only increases the uncertainty of the results. From 5 to 10 c.c. of 10 per cent. barium chloride added drop by drop to the hot sulphate solution in from two to ten minutes may therefore be given as a safe rule for the use of barium chloride in sulphate determinations. In special cases to be described later, 5 per cent. barium chloride must be substituted for the 10 per cent. solution.

IV.—THE PRECIPITATION OF BARIUM SULPHATE IN THE COLD,
AND THE PECULIAR BEHAVIOR OF POTASSIUM
SULPHATE SOLUTIONS.

The fact that beautiful crystalline barium sulphate precipitates can readily be obtained at ordinary room temperatures has already been mentioned. Having once stumbled upon this interesting fact, I naturally thought that it might be made serviceable in quantitative determinations of sulphates. The standard SO_4 solutions at that time in use in this laboratory were made exclusively from potassium sulphate. The results obtained in the cold with these solutions were indeed surprising. The first preliminary determinations showed considerable variations, but after the right conditions had been determined the limit of error sank to less than 0.5 milligram. And the results were equally good whether the filtration was made the day following the precipitation or at the end of fifteen minutes. For example, with a solution, 20 c.c. of which should yield 231.7 milligrams of barium sulphate, the following consecutive figures were obtained: 231.7, 231.7, 231.8, 231.7. The filtrations in this series were made at the end of fifteen minutes. Another solution, the theoretical value of which was 187 milligrams, gave the following consecutive figures: 187.4, 187.3, 187.4.

The precipitations in the above determinations were made by dropping 10 c.c. of barium chloride solution (5 per cent.) into the sulphate solutions, which had been diluted to a volume of 150 c.c., and which contained 2 c.c. of concentrated hydrochloric acid. The sulphate solutions were not shaken or even moved during the addition of the barium chloride.

It is to be noticed that the precipitation just described, which gives perfectly accurate results, differs apparently very little from the precipitation described on page 133, which was there said to give precipitates that weigh too little and lose sulphuric acid when heated. The apparently insignificant, yet in reality exceedingly important, difference between the two is only the shaking of the sulphate solution while the barium chloride is being added. Shaking the solution will lower the result by several milligrams, because the precipitation will then lose sulphuric acid during the ignition. For example, simultaneously

with the last three determinations quoted, three others were made in exactly the same manner, except that the sulphate solutions were shaken during the precipitation. The following figures were obtained: 182.6, 182.8, 184.2. (Theoretical value, 187.) Another series made with shaking gave the following figures: 183.8, 183.3, 182.4, 182.3, 183.1, 184.1. (Theoretical value, 187.) In the presence of much hydrochloric acid the difference due to shaking becomes even more pronounced. Thus with 10 c.c. of concentrated hydrochloric acid the figures obtained were: without shaking, 185.3; with shaking, 177.2. The filtration was made at the end of ten minutes.

If the mere matter of shaking or not shaking the sulphate solution could be of such great consequence when the precipitation is made in the cold, it was thought that it might be a very important detail in precipitating barium sulphate from hot solutions. An actively boiling solution might then give very different results from a merely hot one. This supposition proved incorrect. While investigating it I came across another peculiarity, however, which I did find to be common to both hot and cold potassium sulphate solutions. It is this: *The weight of the barium sulphate precipitates obtained depends to a great extent upon the concentration of the barium chloride solution, no matter how slowly it is added.*

For several months prior to the discovery of this fact I had used potassium sulphate in the preparation of the standard sulphate solutions, and had uniformly used a 10 per cent. concentration of barium chloride for the precipitation. After having carefully scrutinized every detail in the technique, excellent results were obtained, but the figures always showed a tendency to be a trifle low rather than too high, as is the common experience in sulphate determinations. The solutions diluted to 150 c.c. were always thoroughly hot, but not boiling, during the addition of the barium chloride. The precipitations were made in the presence of 1 or 2 c.c. of concentrated hydrochloric acid. The following series of consecutive determinations may be quoted to show that the results, though accurate, tend to be low:

Solution No. 1. Theoretical BaSO_4 in 20 c.c. = 290.8 mgms. Found: (1) 290.4, (2) 290.0, (3) 290.6. Average, 290.3 mgms.

Solution No. 2. Theoretical BaSO_4 in 20 c.c. = 281.6 mgms. Found: (1) 281.3, (2) 281.9, (3) 281.0, (4) 281.1, (5) 281.3, (6) 281.0, (7) 281.1. Average, 281.2 mgms.

Solution No. 3. Theoretical BaSO_4 in 20 c.c. = 336.3 mgms. Found: (1) 336.4, (2) 336.0, (3) 336.1, (4) 335.5, (5) 336.1, (6) 336.0. Average, 336.0 mgms.

The following series of determinations was made in exactly the same manner as those quoted above, except that 10 c.c. of 5 per cent. barium chloride were substituted for 5 c.c. of 10 per cent. barium chloride.

Solution No. 14. Theoretical BaSO_4 in 20 c.c. = 187.0 mgms. Found: (1) 184.9, (2) 186.8, (3) 185.0, (4) 186.4, (5) 186.1, (6) 183.9, (7) 186.1. Average, 185.6 mgms.

In the following three determinations made with the same solution, shaking was used during the addition of the 5 per cent. barium chloride solution:

Found: (1) 185.1, (2) 186.2, (3) 185.1. Average, 185.5 mgms.

Twenty c.c. of 2.5 per cent. barium chloride gave virtually the same result as the 5 per cent. solution.

Found: (1) 185.4, (2) 185.9, (3) 185.7. Average, 185.7 mgms.

With a 15 per cent. solution of barium chloride (5 c.c.), the following figures were obtained: (1) 188.1, (2) 187.8, (3) 186.8. Average, 187.6 mgms.

For the determination of SO_4 in pure potassium sulphate solutions, 10 per cent. barium chloride may be said to give the best results if the precipitation is made near the boiling temperature. Fifteen per cent. barium chloride gives somewhat too high results, and 5 per cent. solutions give results that are decidedly too low. The precipitates obtained with 5 per cent. barium chloride probably lose sulphuric acid during the ignition, but I have not tested this point.

When the precipitation is made in the cold, 5 per cent. barium chloride must be used in order to obtain correct results. Figures bearing this out have already been quoted. With 10 per cent. barium chloride added at room temperature the following figures were obtained: (1) 189.1, (2) 188.2, (3) 188.7. Average, 188.6. Theoretical, 187.0 mgms.

"Dumping" dilute (2 per cent.) barium chloride into cold sulphate solutions gives beautiful crystalline precipitates, but the analytical results obtained by this means are always too high. This is illustrated by the following figures obtained from the same solution: (1) 188.8, (2) 189.4, (3) 188.7, (4) 188.5, (5) 188.7, (6) 189.3, (7) 189.3, (8) 188.3, (9) 188.6, (10) 189.1. Theoretical value, 187.0 mgms.

By the use of 10 per cent. barium chloride in the heat or by the use of 5 per cent. barium chloride in the cold, without shaking or stirring, the SO_4 in pure potassium sulphate solutions may be determined with very great accuracy.

V.—EFFECTS OF POTASSIUM SALTS ON BARIUM SULPHATE DETERMINATIONS.

The analytical figures quoted above show conclusively that the weight, and consequently the chemical composition, of barium sulphate precipitates obtained from potassium sulphate solutions depend upon the concentration of the barium chloride solution used for the precipitation. While this is true in the case of potassium sulphate solutions, it would be a mistake to assume that it is equally true for other sulphate solutions. The rule does not hold for solutions of pure sulphuric acid. They give as accurate results with 5 per cent. as with 10 per cent. barium chloride when the precipitations are made at boiling temperature, and give slightly too high results in the cold whether 5 per cent. or more dilute barium chloride solutions are used for the precipitations. This is shown by the figures cited below. The strength of the sulphuric acid was carefully determined by means of both calc spar and sodic carbonate (using hydrochloric acid and sodic hydrate for the intermediate titrations).

H_2SO_4 Solution No. 12. Theoretical BaSO_4 in 20 c.c. = 150.9 mgms.

In the heat, 10 per cent. barium chloride, found: (1) 151.1 mgms.,
(2) 151.1 mgms.

In the heat, 5 per cent. barium chloride, found: (1) 151.0 mgms.,
(2) 150.8 mgms.

In the cold, 5 per cent. barium chloride, found: (1) 152.5 mgms.,
(2) 152.7 mgms., (3) 152.3 mgms.

In the cold, 3.5 per cent. barium chloride, found: (1) 152.0 mgms.,
(2) 151.5 mgms., (3) 151.9 mgms.

In the cold, 2.5 per cent. barium chloride, found: (1) 152.2 mgms.,
(2) 152.2 mgms.

The presence of but one gram of potassium chloride in the sulphuric acid destroys the accuracy of the determination, whether made in the heat or at ordinary temperatures. One gram of KCl (Kahlbaum's) was added in each of the following determinations.

Volume, as usual, 150 c.c. Concentrated HCl present, 2 c.c. Theoretical BaSO_4 , 150.9 mgms. Found: in the heat (10 per cent. barium chloride), (1) 149.1 mgms., (2) 149.3 mgms.; in the cold (5 per cent. barium chloride), (1) 149.1 mgms., (2) 148.8 mgms.

The peculiar disturbing effects due to the presence of potassium salts offer an interesting field for the study of theoretical chemistry. The deficit of over 1 per cent. found in the presence of one gram of potassium chloride is not due to an increased solvent action of the potassium chloride solution. I have repeatedly satisfied myself that minimum traces of SO_4 are precipitated quite as readily in the presence of potassium chloride as in its absence. The cause of the phenomenon is undoubtedly due to the different chemical composition of the precipitates; to the formation of larger quantities of the salt $\frac{\text{HSO}_4}{\text{HOS}_4} > \text{Ba}$, involving, as was shown at the beginning of this paper, demonstrable losses of sulphuric acid during the ignition. The exhaustive study of the chemistry of the phenomenon does, however, not properly belong to a hospital laboratory. I am concerned only with the best way of getting reliable analytical results.

The practical bearing of the fact that potassium chloride destroys the accuracy of sulphate determinations is by no means unimportant within the domain of physiological chemistry. Nearly all sulphur determinations in protein substances, as well as the total sulphur determinations in urine, are made by the use of large quantities of "fusion mixture," consisting of potassium nitrate and sodium carbonate in varying proportions, yet always containing several times as much potassium as sodium. In all these cases the barium sulphate is precipitated in the presence of very large quantities of potassium chloride.

The conclusion seems warranted that if any of these determinations are correct it is largely a matter of good luck. Most of them must be unreliable.

The disturbing effects of potassium chloride can be counteracted by adding an excess of sodium chloride. Four grams of the sodium salt for each gram of the potassium salt are necessary to accomplish this result. This is shown in the following determinations made with sulphuric acid solution No. 12. In these, as in all the determinations not otherwise described, the precipitation has been made in the heat with 10 per cent. barium chloride (5 c.c.).

Theoretical BaSO_4 in 20 c.c. = 150.9 mgms.

Present 1 gm. KCl , 1 gm. NaCl . Found, 149.6 mgms.

"	1	"	"	3	"	"	150.3	"
"	1	"	"	5	"	"	151.2	"
"	1	"	"	8	"	"	151.6	"
"	1	"	"	4	"	"	(1) 151.0 mgms., (2) 151.2 mgms., (3) 150.9 mgms.	
"	2	"	"	8	"	"	(1) 151.3 mgms., (2) 150.9 mgms., (3) 151.2 mgms.	
"	0	"	"	8	"	"	(1) 151.1 mgms., (2) 151.0 mgms.	

On the basis of these findings it should not be very difficult to prepare a hydrate-nitrate fusion mixture suitable for total sulphur determinations in urine and protein substances. To do so seems, however, superfluous in view of the admirable qualities of sodium peroxide. The use of this substance for total sulphur determinations in urine will be described below.

VI.—THE INFLUENCE OF SOME OTHER SUBSTANCES (NH_4Cl , NiCl_2 , NaCl , Na_2SO_4 , KNO_3 , NaNO_3 , AND HCl) ON THE BARIUM SULPHATE PRECIPITATES.

The facts brought out in connection with the determination of SO_4 in potassium sulphate and in sulphuric acid with and without the presence of potassium chloride show how easily errors can creep into "practical" sulphate determinations made under no well defined conditions. The experiments recorded on the next three pages (144-147) were made chiefly to throw light on the conditions that prevail in urine analysis.

Ammonium Chloride.—The addition of ammonium chloride is one of the "tricks" that has often been recommended to facilitate the formation of coarsely granular precipitates which can easily be filtered. That precipitates suitable for filtration can be obtained without any such accessories has already been shown. With regard to ammonium chloride, I have found that it is not entirely without influence on the analytical results obtained. Its effects are similar to those of potassium chloride, though much less pronounced.

K_2SO_4 Solution No. 8. Theoretical $BaSO_4$ in 20 c.c. = 336.5 mgms. Ammonium chloride 0 gms. Found: (1) 335.9, (2) 336.1, (3) 336.3, (4) 336.9, (5) 335.9, (6) 335.9.

Ammonium chloride 10 gms. Found: (1) 334.7, (2) 334.1, (3) 334.6.

Nickelic Chloride.—When sodium peroxide is used to oxidize organic substances in nickel dishes the latter nearly always give off small amounts of nickelic oxide. To remove this oxide by filtering and washing is a very tedious operation, especially on account of the large amounts of alkali present. It therefore seemed important to determine whether the corresponding amounts of nickelic chloride interfere with correct sulphate determinations. This seemed all the more necessary in view of the fact that ferric salts are known to interfere. A weighed piece of nickel (from a nickel dish) was consequently converted into nickelic chloride and made up to a known volume. Twenty-five c.c. of this solution, containing 0.375 gram of $NiCl_2$, were added in each case.

K_2SO_4 Solution No. 1. Theoretical $BaSO_4$ in 20 c.c. = 290.8 mgms. Found: (1) 290.1, (2) 290.3, (3) 290.7.

The same amount of nickel together with 9 grams of sodium chloride gave 292.0 and 290.9 milligrams of barium sulphate.

Neither alone nor in the presence of sodium chloride can any disturbing effects be traced to the presence of moderate quantities of nickel chloride.

Sodium Chloride.—It was shown on page 143 that an excess of sodium chloride counteracts the disturbing effects of potassium chloride. That this counteracting influence of sodium chloride is not due to any tendency in the sodium salt to produce

too high results is shown by the last experiment of the series. In the presence of 8 grams of sodium chloride alone (*i.e.*, without any KCl), the correct figure was obtained. This point needs further verification on account of the large quantities of sodium chloride necessarily present in many sulphate determinations. The following six determinations were made in the presence of 10 grams of NaCl with potassium sulphate solution No. 2, containing 281.6 milligrams of BaSO_4 in 20 c.c.:

Found: (1) 280.6, (2) 280.8, (3) 282.4, (4) 280.7, (5) 280.9, (6) 280.3.
Average, 280.9 mgms.

Sulphuric acid solution No. 12 gave almost equally satisfactory results in the presence of 10 grams of sodium chloride and 5 c.c. of concentrated hydrochloric acid (water present, 250 c.c.).

Theoretical value, 150.9 mgms. BaSO_4 . Found: (1) 151.3, (2) 151.7, (3) 151.2, (4) 150.8. Average, 151.2 mgms.

Sodium Sulphate.—Since potassium sulphate and sulphuric acid do not give perfectly equivalent barium sulphate figures when treated under similar conditions with barium chloride, it seemed worth while to make a few determinations with sodium sulphate. This salt ought to give results strictly equivalent to those given by sulphuric acid, since the latter gives theoretical figures in the presence of sodium chloride. The results actually obtained were, however, uniformly too high. Kahlbaum's purest sodium sulphate was used. The first solution was made from a sample of the salt which had been heated to dull redness in a platinum dish for about half an hour. 5.9760 gms., dissolved and diluted to 2 liters, gave a solution, 20 c.c. of which should yield 98.15 mgms. BaSO_4 . Only two determinations were made, and in these the precipitations were made in the cold (5 per cent. barium chloride). The first gave 99.7, the second 99.7 mgms. barium sulphate. Thinking that the salt, though neutral to litmus, might contain traces of the bisulphate, a second and smaller quantity was heated to a distinct red heat for fully two hours. 3.5979 gms. were dissolved and diluted to one liter. Twenty c.c. should yield 118.2 mgms. BaSO_4 . Two determinations made in the heat gave 119.1 and 118.9 mgms. BaSO_4 . Two parallel determinations in the cold gave 119.2 and 119.2 mgms. Of course I have no guarantee of the purity of the salt used, but the fact that the results are high, and not low, practically excludes every impurity except lithium sulphate.

Sodium and Potassium Nitrate.—It has long been known that nitrates interfere with sulphate determinations. The following figures are quoted merely to indicate the degree of error due to the presence of these salts:

K_2SO_4 Solution No. 1. Theoretical $BaSO_4 = 290.8$ mgms. Found in the presence of 10 gms. $NaNO_3$ (150 c.c. H_2O): (1) 301.1, (2) 304.3, (3) 304.6.

10 gms. KNO_3 gave 235.0 mgms. $BaSO_4$, where the theoretical value was 231.7. Sodium nitrate gives, therefore, as was to be expected, greater errors than potassium nitrate. The potassium of the latter evidently counteracts in part the action of the acid radical.

Hydrochloric Acid.—The presence of a certain amount of strong uncombined acid is indispensable for correct sulphate determinations. This fact was overlooked by Baumann when he prescribed the substitution of acetic for hydrochloric acid for the determination of the inorganic sulphates in urine. His method gives higher figures for the inorganic sulphates than the sum of both the inorganic and ethereal sulphates present. Adding neutral barium chloride (10 per cent. solution) in the usual manner to a neutral hot potassium sulphate solution (theoretical value, 281.6 mgms.) gave the following figures: (1) 285.4, (2) 285.4, (3) 285.4, (4) 285.4. Adding 8 c.c. of concentrated hydrochloric acid a few minutes *after* the addition of barium chloride gave the following figures with the same solution: (1) 285.8, (2) 283.3, (3) 284.3, (4) 284.2. Adding only 1 c.c. of HCl to the solution containing the precipitated barium sulphate gave the following: (1) 284.4, (2) 284.9, (3) 284.5, (4) 283.9, (5) 283.9, (6) 283.1, (7) 282.7, (8) 283.2. In the last four determinations the precipitate had stood for twenty hours before the filtration was begun.

How much hydrochloric acid can be present without lessening the accuracy of the results? In all the "standard" determinations recorded in this paper 1 or 2 c.c. of concentrated hydrochloric acid was present, together with about 150 c.c. of water. It has generally been assumed that too much hydrochloric acid leads to too low barium sulphate figures, because barium sulphate is more soluble in acids than in water. Some careful investigators have attempted to meet this difficulty by always using the same concentration of hydrochloric acid, and then making a correction for the solubility of barium sulphate. This procedure is, of course, based on the erroneous assumption that the precipitates consist of pure $BaSO_4$. The importance of the hydrochloric acid lies, not so much in its effect on the solubility

of the precipitates, as in its effect on their chemical composition. Richards (*loc. cit.*) was, I believe, the first to point out that the "occlusion" of barium chloride is very much increased in the presence of large amounts of hydrochloric acid. The following experiments show that the increased "occlusion" is more than sufficient to make up for the increased solubility of the precipitates when much hydrochloric acid is present:

K₂SO₄ Solution No. 2. Theoretical BaSO₄, 281.6 mgms. H₂O present, 150 c.c.; HCl present, 8 c.c. Found: (1) 285.8, (2) 283.3, (3) 284.3, (4) 284.2, (5) 282.0, (6) 283.1, (7) 284.6, (8) 283.3. Average, 283.8 mgms.

K₂SO₄ Solution No. 1 (theoretical BaSO₄, 290.8 mgms.) gave the following figures when the barium chloride was added by means of a pipette (HCl present, 10 c.c.): (1) 295.1, (2) 294.2, (3) 295.3. Average, 294.9 mgms.

On the basis of the experience gained in this work I should say that from 1 to 4 c.c. of concentrated hydrochloric acid in 150 c.c. of solution represent the safe limits of acidity for accurate barium sulphate determinations. When for any reason more hydrochloric acid has been added, a simple and safe remedy is to dilute with water before adding the barium chloride.

VII.—GOOCH CRUCIBLES *versus* FILTER PAPER IN SULPHATE DETERMINATIONS.

Filter paper offers one great advantage. It enables one to make a whole series of simultaneous filtrations and washings, which saves time. But it is, I believe, practically impossible to make as accurate sulphate determinations with the help of filter paper as can be obtained with Gooch crucibles. This statement may be verified by making two series of five or six determinations, using on the one hand filter paper, on the other hand Gooch crucibles. It will be found, I think, that the filter paper series will show greater variations and a lower total average than the Gooch crucible series. The error due to the use of filter paper was discovered in connection with the potassium sulphate work. In the endeavor to find the reason why the results were always low, which seemed so contrary to all common experience with sulphate determinations, every detail

of the manipulation was subjected to critical examination. When Gooch crucibles were substituted for filter paper, it was found that the results became both more uniform and enough higher to give practically theoretical figures.

It seems to me that the reason why losses during the burning of the filters have not been noticed before must be that nearly all careful work has been made with sulphuric acid solutions, and under conditions that should give too high results. One error has been made to compensate for another.

It has indeed been pointed out that some error may creep in in connection with the burning of the filters, but this error has been explained as a reduction of sulphate to sulphide. Occasionally this may happen, but not often. It is seldom that barium sulphate precipitates gain in weight after treatment with a drop of sulphuric acid and reheating. The mechanical loss of notable quantities of barium sulphate occurs, however, nearly every time. Even in the slow charring process recommended by Richards, when the filter is at no time allowed to take fire, there is a mechanical loss of about 1 milligram of barium sulphate. The best results which I have been able to obtain by the charring process may be given here:

Theoretical BaSO_4 , 290.8 mgms.

Found (using filters): (1) 289.4, (2) 289.9, (3) 289.3. Average, 289.5 mgms.

Found (using Gooch crucibles): (1) 290.4, (2) 290.0, (3) 290.6. Average, 290.3 mgms.

When the filters are allowed to "burn," the mechanical losses incurred are very much greater (from 1 to 4, or even 5, milligrams).

All the determinations cited in this paper (except the three just quoted) have been made with the help of Gooch crucibles. Only porcelain crucibles have been used, as those made of platinum are very expensive. It may be permissible to describe here the method of using these crucibles, and to point out one source of error which must be avoided in order to obtain reliable results.

The asbestos for the mats must be of good quality, consisting chiefly of long shiny fibres. It is perhaps most easily prepared for use as fol-

lows: The fibres are cut with scissors into suitable lengths (50–70 mm.). A few grams at a time are then placed in a cylinder with about 300 c.c. of 5 per cent. hydrochloric acid, and a strong air current is passed through for a few minutes. This separates all the fibres far more quickly and completely than the usual method of scraping the fibres with a knife. In an hour or two asbestos enough for two hundred crucibles can be prepared. It is kept ready for use in dilute hydrochloric acid. From 50 to 100 mgms. of asbestos is used for each mat. By using a good vacuum pump at almost full force the asbestos mat is packed into a thin, but uniform and firm layer in the bottom of the crucible. It is finally washed by the help of only enough of a vacuum to make the water run through in a slow stream; it is then dried, ignited, and weighed. Mats so prepared are as effective as the best filter paper in retaining precipitates, and there is practically no danger of losing any asbestos during the subsequent washings of barium sulphate precipitates. The same mat can advantageously be used until about 1 gram of barium sulphate has collected. Time is saved by not using the same mat too long, because the filtration becomes slower and slower the more precipitate there is present, and it is not safe to increase the vacuum too much.

The ignition of the precipitates is associated with more serious sources of error than the filtration, more serious because they are not accessible to direct observation: The flame must not be applied directly to the perforated bottom of the crucibles. If this is done mechanical losses are sure to occur, even though the crucibles are covered with lids. Nor is it safe to apply the flame to the sides of the crucibles. To do so involves again mechanical loss of barium sulphate. During the ignition the crucibles must be provided not only with lids, but also with tight bottoms. This is easily accomplished by the use of lids of ordinary platinum crucibles. The lid is placed on a triangle, and the crucible stands in upright position on top, while the flame is applied to the platinum lid. These points may seem trivial, but they consume no extra time, and they are necessary for uniformly reliable figures. Ten minutes' ignition is sufficient, unless organic matter is present.

VIII.—THE FILTRATION AND WASHING OF BARIUM SULPHATE PRECIPITATES.

In this laboratory all barium sulphate precipitates are filtered cold. Formerly we used very hot water for the washing of precipitates. Since it was found that sulphates can be determined

without heat during the precipitation, hot water has also been discarded for the washing of precipitates. Cold water serves the purpose equally well, is more convenient, and is free from some disagreeable features associated with the use of hot water. When very hot water is poured on a barium sulphate precipitate in a flask or beaker, the precipitate visibly attaches itself to the glass. When this has once happened it is extremely difficult to remove it all. Thorough rubbing over every part may loosen it, but it is then of the "creeping" kind and does not wash out. Even though the flask after much rubbing and rinsing appears perfectly clean and is then set aside, it will be found after an hour's standing to contain noticeable "streaks" of barium sulphate. This evil is very much reduced when cold water is used, and is entirely absent when the sulphate determinations are made altogether in the cold, because the filtration can then be begun before the precipitate has settled. Hot water will occasionally spoil a determination when Gooch crucibles are used, because it leads to sudden expansion of the air in the flask, sufficient to loosen the asbestos mat in the crucible.

IX.—THE DETERMINATION OF SULPHATES AND TOTAL SULPHUR IN URINE.

The investigations described in this paper are the outcome of a conviction that the published records of sulphate and sulphur determinations in urine, including many of my own, are intolerably unreliable. The extremely erratic figures ascribed to the "neutral" sulphur and the large variations in published "duplicate" determinations constitute positive evidence, it seems to me, that there is something wrong about the technique. On the pages that follow will be given descriptions of the determinations as they are now carried out in this laboratory. I have endeavored to work out conditions which shall make it possible to make a whole series of parallel determinations within a maximum variation of one per cent. I believe that any one who will carefully observe the conditions described below, without introducing any untried variations, will find that this purpose has been accomplished.

1. *Inorganic Sulphates*.—Only one method has ever been

proposed for the direct determination of inorganic sulphates, namely that of Baumann,¹ advanced in 1877. This method is based on the fact that the ethereal sulphates are not split by hot dilute acetic acid, and on the assumption that the inorganic sulphates can be determined in dilute acetic acid solutions. Baumann evidently did not test the accuracy of the latter assumption, or he would certainly have found how grossly erroneous it is when applied to urine. The method seems never to have received any serious attention, although descriptions of it are still found in some text-books. It has been entirely replaced by the procedure of Salkowski, which gives the inorganic sulphates as the difference between the total and the ethereal sulphates.

The specific practical point which I had in view in studying the behavior of cold sulphate solutions when precipitated with barium chloride was to find a suitable method for the direct determination of inorganic sulphates in urine.

The method is as follows: About 100 c.c. of water (not less), 10 c.c. of dilute hydrochloric acid (1 part concentrated HCl to 4 parts H₂O by volume), and 25 c.c. urine are measured into an Erlenmeyer flask (capacity 200–250 c.c.). If the urine is dilute, 50 c.c. instead of 25, and a corresponding, smaller quantity of water, may be taken. Five per cent. barium chloride solution (10 c.c.) is then added, always drop by drop, preferably by means of an automatic dropper. The urine solution is not to be shaken, stirred, or otherwise disturbed while the barium chloride is being added. At the end of an hour or later, according to convenience, the mixture is shaken up and filtered through a Gooch crucible. The precipitate is washed with about 250 c.c. cold water, dried, and ignited according to the directions given on page 149.

A few series of sulphate determinations made as above with mixed human urine may be cited, although they prove only that the method gives concordant results:

Urine No. 1. (Sp. gr. 1.028.) BaSO ₄ found in 25 c.c.:									
(1)	169.2	mgms.	(Filtered at the end of 15 minutes).						
(2)	169.8	"	(" " " " " " 30 ")						

¹ Baumann, *Zeitschrift für physiologische Chemie*, i, p. 77, 1877.

(3) 169.9 mgms. (Filtered at the end of 35 hours).

(4) 168.6 " (" " " " " 48 ").

Urine No. 2. (Sp. gr. 1.025.) Mgms. of BaSO_4 in 50 c.c.:

(1) 207.8, (2) 208.5, (3) 207.4, (4) 207.1, (5) 207.5, (6) 206.8, (7) 207.4.

Baumann's method applied to urine No. 2 gave 239.9 and 238.1 mgms.

Urine No. 4 (dilute cystinuria urine). Mgms. of BaSO_4 found in 50 c.c.:

(1) 116.2, (2) 116.4, (3) 116.2, (4) 116.1, (5) 116.1.

Urine No. 5. Mgms of BaSO_4 in 50 c.c.: (1) 167.8, (2) 168.8, (3) 168.1,

(4) 168.2, (5) 168.9, (6) 168.6.

I believe that the procedure described above will be found very satisfactory for urine work. The precipitates are almost completely free from organic matter, and the filtrates are clear. The precipitation is complete in a very short time so that the filtration can be begun with perfect safety one hour after the addition of the barium chloride. Yet if convenience demands it, there is no danger in waiting longer before filtering. The strength of acid used does not split the ethereal sulphates at ordinary room temperatures. I have satisfied myself of this point partly by saving the filtrates—they remain clear—and partly by waiting twenty-four hours before filtering without obtaining higher figures. Nothing is gained, however, by waiting with the filtration. The precipitate need not have "settled"; on the contrary, it is advantageous to shake up the solution at the beginning of the filtration and occasionally during it. If this is done, two or three subsequent rinsings are sufficient to transfer the last remnants of the precipitate from the flask to the filter. The results obtained are very uniform, and are more accurate, I believe, than those of any other known method. All urines contain, of course, some potassium salts, which might tend to render the results a little low. But the sodium salts present seem to be sufficient to counteract the potassium salts. (I have not been able to obtain higher results by adding sodium chloride.)¹

2. *Total Sulphates* (Inorganic + Ethereal).—The total sulphates in urine may be precipitated either in the heat or in the cold. I prefer the latter as it is more convenient, but will de-

¹ It might be advantageous, before precipitating with barium chloride, to add one or two grams, sodium chloride to urines which may be supposed to contain unusual quantities of potassium.

scribe both. Whether the precipitation is made in the heat or in the cold certain precautions not mentioned in text-books must be observed, if correct and concordant figures are to be obtained. The treatment with hot, dilute hydrochloric acid must be vigorous enough to split with certainty all the ethereal sulphates (that can be split by acid treatment); yet too much hydrochloric acid must not be used because it seriously alters (as has already been shown, p. 147) the chemical composition of barium sulphate precipitates.

The present basis for total sulphate determinations is the investigations of Salkowski¹ which showed that fifteen minutes' boiling of urine, diluted to 100 c.c., with 10 c.c. of hydrochloric acid (sp. gr. 1.12), splits all the ethereal sulphates. Unfortunately this amount of hydrochloric acid (equal to 6 c.c. HCl, sp. gr. 1.20) destroys the accuracy of the subsequent barium sulphate precipitation. The procedure as described by Salkowski is liable to give results that are several milligrams too high. To overcome the difficulty, it is not safe to reduce the amount of hydrochloric acid in proportion to the amount of urine taken. More acid than that recommended by Salkowski, rather than less, and longer boiling, are needed to be quite sure of splitting all the ethereal sulphates. Suitable conditions are obtained by using less urine than Salkowski recommends (never more than 50 c.c.) and by omitting to dilute it to 100 c.c. during the boiling. The dilution should come after the boiling.

Either of the following two procedures gives, I think, perfectly reliable results for the total sulphates of urine.

a. *Barium Sulphate Precipitation in the Cold.*—Twenty-five c.c. of urine and 20 c.c. of dilute hydrochloric acid (1 part HCl, sp. gr. 1.20, to 4 parts H₂O), or 50 c.c. of urine and 4 c.c. of concentrated hydrochloric acid, are gently boiled in an Erlenmeyer flask (capacity 200–250 c.c.) for 20 to 30 minutes (not less than 20). To reduce the loss of steam it is better to keep the flask covered with a small watch glass during the boiling. The flask is cooled for two or three minutes in running water, and the contents are diluted with cold water to about 150 c.c. To this cold solution is then added 5 per cent. barium chloride

¹ Salkowski, *Zeitschrift für physiologische Chemie*, x, p. 346, 1886.

(10 c.c.) without any shaking or stirring during the addition. The remainder of the procedure is like that of the inorganic sulphate determination described on page 151.

b. *Barium Sulphate Precipitation in the Heat*.—The boiling of the urine with hydrochloric acid is conducted exactly as in the preceding method. At the end of 20 to 30 minutes the boiling urine is diluted to about 150 c.c. with hot water. The mixture is heated once more to the boiling point, is then taken off the fire, and at once precipitated with ten per cent. barium chloride solution (5 c.c.). The barium chloride must always be added drop by drop. The filtration is made after about two hours' standing, when the mixture has acquired the room temperature. The remainder of the procedure is like that of the inorganic sulphate determination.

A few total sulphate determinations may be quoted:

Urine No. 2. (Sp. gr. 1.025.) Mgms. of BaSO_4 in 25 c.c. as found by the first procedure (in the cold): (1) 221.6, (2) 221.8, (3) 222.0, (4) 221.8; as found by the second procedure (in the heat): (1) 220.8, (2) 221.3, (3) 220.7, (4) 222.3, (5) 222.0.

Urine No. 3. Mgms of BaSO_4 in 50 c.c.: By the first procedure, (1) 237.2, (2) 237.4. By the second procedure: (1) 237.0, (2) 236.6, (3) 237.0, (4) 236.9.

Urine No. 4. (Filtered cystinuria urine.) Mgms. of BaSO_4 in 50 c.c.: By the first procedure, (1) 123.4, (2) 124.2, (3) 123.5, (4) 123.5, (5) 123.8, (6) 124.0.

Urine No. 5. Mgms of BaSO_4 in 50 c.c.: (1) 181.9, (2) 182.2, (3) 182.8, (4) 182.9, (5) 182.9, (6) 182.9.

3. *Ethereal Sulphates*.—I believe that the methods described for the determination of the inorganic and total sulphates give exceedingly accurate figures, and that the ethereal sulphates are represented with a high degree of accuracy by the difference between the two. A few remarks concerning the direct determination of ethereal sulphates may, however, not be superfluous.

Salkowski's method for the determination of ethereal sulphates has for a long time been the only one in use. The chief point of the method is the precipitation of the inorganic sulphates in the cold by means of a mixture of barium chloride and barium hydrate. The use of the hydrate has always appeared to me an objectionable as well as unnecessary feature, and in

my own determinations I have always used barium chloride alone. Kossel¹ raised an objection to the use of barium hydrate as far back as 1882, on the ground that such an alkaline barium mixture might carry down sulphate compounds which properly belong to the ethereal sulphate group. Salkowski, however, did not concede the pertinence of the objection (*loc. cit.*, p. 348), and the matter was allowed to drop.

The validity of Kossel's objection to the use of Salkowski's alkaline barium chloride mixture for the precipitation of the inorganic sulphates can now easily be tested by means of the method described above for the precipitation of the inorganic sulphates in the presence of free hydrochloric acid. The matter seems no longer to be of much importance, because the direct determination of ethereal sulphates can advantageously be replaced, I think, by the direct determination of the inorganic sulphates. Only one determination has therefore been made, but it shows that Salkowski's alkaline barium chloride mixture does carry down some ethereal sulphate compounds. The alkaline filtrate representing 50 c.c. of a mixed concentrated urine, obtained according to Salkowski, gave 13.3 and 13.1 milligrams of barium sulphate. Two similar filtrates, obtained after precipitating the inorganic sulphates in the presence of hydrochloric acid, gave 15.3 and 15.0 milligrams of barium sulphate.

When much urine is available the direct ethereal sulphate determination may be made as follows:

One hundred and twenty-five c.c. urine are diluted with 75 c.c. of water and 30 c.c. of dilute hydrochloric acid (1:4). The solution is precipitated in the cold by the addition of 20 c.c. of five per cent. barium chloride, the addition being made by means of a dropper, as previously described. After one hour's standing the mixture is filtered on a dry filter, and 125 c.c. of the filtrate are gently boiled for not less than thirty minutes, allowed to cool filtered, washed, and ignited as usual.

4. *Total Sulphur*.—The determination of the total sulphur in urine has proved by far the most difficult task encountered in the course of these investigations. Several times I have thought that the right procedure was found, only to be disillusioned by the results of subsequent determinations. The problem was to

¹ Kossel, *Zeitschrift für physiologische Chemie*, vii, p. 292, 1882.

find a method, based on the use of sodium peroxide, by means of which several (4-6) parallel determinations could be made within a variation of not over 1 per cent. of the total. The making of such prolonged series constitutes a very severe test of the certainty of any analytical method, but it brings out its weak points. As far as I know, no one has systematically applied this test to the determinations of the total sulphur in urine. Modrakowski,¹ one of the first to use sodium peroxide for total sulphur determinations in urine, appears not even to have made duplicate determinations. To be sure, he made parallel determinations by means of fusion mixtures made from saltpetre and sodium carbonate, and records quite good agreement between the two sets of results. Unfortunately, however, Modrakowski has thereby only proved that the sodium peroxide method which he describes gave in his own hands results which cannot be correct. His "standard" results obtained by the help of the saltpetre fusion mixture are undoubtedly erroneous on account of the presence of potassium chloride.

The most apparent, though by no means the most serious, difficulty connected with the use of sodium peroxide for the oxidation of the organic matter in urine is the conflagration. The mechanical loss of sulphates due to this cause is practically negligible, provided that sufficient quantity of peroxide is used; but the conflagration must be avoided because it leads to the formation of substances which interfere with the subsequent precipitation of barium sulphates. Nitrates are invariably formed when dried urine residues are burned by means of sodium peroxide. The solutions which should be ready for the addition of barium chloride show strong oxidizing properties which do not disappear on boiling. They decolorize methyl orange and oxidize alcohol to aldehyde. These difficulties are overcome by providing for the presence of a certain amount of moisture in the urine residue before adding the peroxide. The oxidation of the alkaline residue from 25 c.c. of urine by means of 6 or 7 grams of peroxide is a perfectly simple and safe operation, provided that a little water is added to the residue before adding the peroxide.

¹ Modrakowski, *Zeitschrift für physiologische Chemie*, xxxviii, p. 362, 1903.

The chief difficulty associated with the use of sodium peroxide for total sulphate determinations in urine is the fact that the barium sulphate is frequently not quantitatively precipitated at the end of twenty-four hours' standing. Only after having discovered this fact and after having allowed all the barium sulphate precipitates to digest in the cold for two days before filtering have the results been uniformly satisfactory. Why this is so, and whether the difficulty is inherent in the method or can be overcome by some change in the manipulations I do not know, but I am sure of the fact. It is not safe to filter until the second day after the addition of barium chloride.

One other point should perhaps be discussed in some detail before the method is concisely described as a whole. Large nickel crucibles having a capacity of 200–250 c.c. are, I think, most suitable for use in this determination. They have the following advantages: The bottoms are flat. This hastens the evaporation of the urine and makes the crucibles stand like beakers. The great depth of the crucibles reduces all mechanical losses to a minimum, and protects the crucible contents from contamination with sulphur dioxide (from the gas) during the ignition. The capacity of the crucibles, finally, is so large that the peroxide remaining after the ignition can be completely decomposed by digestion with hot water before transferring the contents to a flask, which avoids the risk of contamination with silicates.

The determination of total sulphur by means of sodium peroxide is as follows:

Twenty-five c.c. of urine (or 50 c.c. if very dilute) is measured into a large nickel crucible (capacity 200–250 c.c.), and about 3 grams of sodium peroxide is added. The mixture is evaporated to a syrupy consistency, and is then carefully heated until it solidifies. This heating may seem a little slow, requiring about fifteen minutes; but the conditions have purposely been selected to make it slow (by using as much as 3 grams of Na_2O_2), in order to drive off as much ammonia as possible before the final fusion with more peroxide. The crucible is removed from the flame and allowed to cool. The residue is then moistened with 1–2 c.c. of water, and, after sprinkling about 7 grams of sodium peroxide over the contents in the

crucible, the mixture is heated to complete fusion for about ten minutes.¹ After cooling for a few minutes, water is added to the contents in the crucible, and the mixture is heated for at least half an hour with about 100 c.c. of water to dissolve the alkali and to decompose the sodium peroxide. The mixture is next rinsed into an Erlenmeyer flask (capacity 400-450 c.c.) by means of hot water, and diluted to about 250 c.c. Concentrated hydrochloric acid is slowly added to the almost boiling solution until the nickelic oxide just dissolves (about 18 c.c. of acid to 8 grams of peroxide). After a few minutes' boiling the solution should be perfectly clear. If it is not clear too much water or too little peroxide has been added for the final fusion. The insoluble residue must then be removed by filtration (after cooling), because it will not dissolve on adding more hydrochloric acid, and too much acid must be avoided. The difficulty does not arise if little water and 7 or 8 grams of peroxide are used.

To the clear acid solution are added 5 c.c. of very dilute alcohol (1 part alcohol to 4 parts H_2O), and the boiling is continued for a few minutes. The alcohol removes the last traces of chlorine, which is always formed on acidifying the solution.² Ten per cent. barium chloride solution (10 c.c.) is next added as usual (by means of a dropper), and the solution is left standing in the cold for *two days* before filtering. The rest of the procedure is the same as for the other sulphate determinations.

A few illustrative analyses may be quoted:

Pure cystin solution in N/10 HCl; 20 c.c. = 76.4 mgms. cystin = 148.5 mgms. $BaSO_4$.

Found: (1) 148.9, (2) 149.3, (3) 148.8, (4) 148.6, (5) 148.2. Average, 148.7 mgms.

Urine No. 2. Mgms. of $BaSO_4$ found in 25 c.c.:

After six hours' standing: (1) 136.0, (2) 137.6, (3) 133.8, (4) 134.1. Average, 135.4.

¹ The addition of a little, but only a little, water before the final fusion is not to be omitted. It not only makes it possible to obtain complete fusion by the aid of comparatively little heat, but it protects the crucible against corrosion, almost completely preventing the occurrence of nickel in the final solution.

² If a few drops methyl orange solution be added at this point, the red coloration which ensues should remain permanent.

After twenty-four hours' standing: (1) 139.5, (2) 138.4, (3) 139.9, (4) 140.3, (5) 139.3, (6) 139.8, (7) 139.9, (8) 139.0. Average, 139.5.
Again after twenty-four hours' standing: (1) 139.5, (2) 139.8, (3) 138.0, (4) 138.2. Average, 138.9.
After forty-eight hours' standing: (1) 140.3, (2) 139.3, (3) 140.1, (4) 139.6, (5) 140.0. Average, 139.9.

The difference between the results as obtained after twenty-four and after forty-eight hours was not very marked in the case of the above urine. The next urine shows the difference in a more unmistakable manner:

Urine No. 3. Mgs. of BaSO_4 found in 25 c.c.:
After six hours' standing: (1) 117.3, (2) 117.6, (3) 118.1, (4) 118.3. Average, 117.8.
After twenty-four hours' standing: (1) 124.3, (2) 124.7, (3) 124.4, (4) 123.4, (5) 126.2, (6) 125.6, (7) 126.2. Average, 125.0.
After forty-eight hours' standing: (1) 128.9, (2) 129.8, (3) 129.3, (4) 128.7, (5) 128.8, (6) 129.6, (7) 128.7. Average, 129.1.
Urine No. 4. (Filtered cystinuria urine.) Mgs. of BaSO_4 found in 50 c.c.: (1) 175.8, (2) 175.9, (3) 175.7, (4) 174.8, (5) 174.8, (6) 176.1, (7) 175.3, (8) 175.7.
Urine No. 5. BaSO_4 found in 25 c.c.: (1) 108.5, (2) 108.8, (3) 109.6, (4) 109.1, (5) 108.8.

In connection with my metabolism publications I have from time to time brought out little modifications for use in sulphate determinations in urine. I always realized that these were inadequate, but have not before had time to thoroughly investigate the subject. These several modifications I now can no longer advocate, except in so far as they coincide with the conditions described in this paper. The total sulphur determination described in the *American Journal of Physiology* (ix, p. 272, 1903), gives very uniform but slightly too low results when sodium carbonate is substituted for the potassium carbonate there recommended.

In conclusion I wish to acknowledge the invaluable services of my assistant, Mr. Christian Östergren. The work has frequently been very tedious as well as discouraging, but his patience and scrupulous care have never failed.

THE CONCENTRATION OF ANTITOXIN FOR THERAPEUTIC USE.

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The Department of Health of New York City since July 1st has been using extensively, and lately exclusively, an antitoxic fluid for diphtheria prepared by a concentration and purification of antitoxic serum. Accordingly some statement as to the nature of the product is desirable. A description of the process may serve the additional purpose of drawing attention to a subject which has of late been somewhat in the background. Both difficulties in the technique of handling the blood proteids and the general confusion of our knowledge of the serum globulins have discouraged to some extent the practical application of proteid chemistry to the concentration of diphtheria and tetanus antitoxins. That an artificial concentration is practicable has been experimentally demonstrated in this laboratory, where such a process has become a part of the routine work in the production of antitoxins.

The serum proteids precipitable by saturation with magnesium sulphate or by half-volume saturation with ammonium sulphate are of three general types—the fibrinogen, the eu- or more typical globulin, and the water-soluble or pseudoglobulin. Some confusion exists as to whether the term "euglobulin" is to be considered as that portion of the serum proteids (exclusive of the fibrin precursors) precipitated by saturation with sodium chloride alone, or to that thrown out of solution by 36 per cent. volume saturation with ammonium sulphate—a concentration of the salt sufficing, according to Pick,¹ to differentiate chemically certain of the antitoxins and other immune substances

¹ E. P. Pick, *Beiträge z. Chem. Physiol. u. Path.*, i, p. 351, 1901.

associated with or actually comprising the serumglobulins. All the evidence at hand is in favor of the serumglobulin nature of antitoxins and some of the related bodies. The antitoxin of diphtheria developed by the immunization of the horse has every character of the soluble globulin in the serum from this animal. A concentration and purification of the antitoxic substance, at least until proteid chemistry is much farther advanced, must, therefore, be based on the separation of this soluble globulin.

The more recent attempts to characterize and differentiate individual serumglobulins have as a basis the association of various immune substances with the proteid precipitates obtained under certain conditions. Thus Belfanti and Carbone¹ found that diphtheria antitoxin was carried down in the globulins obtained by salting out with ammonium and magnesium sulphates, but not with the precipitates obtained by acetic acid. Dieudonné² had previously shown that the proteids thrown out of solution by acetic and carbonic acids contained none of the antitoxin. Seng³ found that diphtheria antitoxin is precipitated along with the soluble globulins. Atkinson⁴ in this laboratory saturated with sodium chloride a solution of the moist serumglobulin precipitate obtained with magnesium sulphate, and by then employing heat differentiated the proteid into several fractions, all of which contained antitoxin; the protective properties corresponded quantitatively to the serumglobulin precipitates. Alteration of the proteid in the fractions by the addition of more of the sulphate produced proportionate changes in the distribution of the antitoxin. Brodie⁵ had previously carried out experiments somewhat similar to Atkinson's with similar results. Pick,⁶ on the contrary, divided the serumglobulin into two parts by ammonium sulphate frac-

¹ Belfanti and Carbone, *Centralbl. f. Bakteriöl.* (Ref.), xxiii, p. 906, 1898.

² Dieudonné, "Ergebnisse der Sammelforschung über das Diphtherie Heilserum," *Arbeiten aus dem Kaiserlicher Gesundheitsamt*, xiii, p. 293, 1897.

³ Seng, *Zeitschr. f. Hyg.*, xxxi, p. 513, 1899.

⁴ Atkinson, *Journ. of Exper. Med.*, v, p. 67, 1901, and some unpublished experiments; see also Park, *Archives of Pediatrics*, Nov. 1900.

⁵ Brodie, *Journ. of Path. and Bact.*, iv, p. 460, 1897.

⁶ Pick, *loc. cit.*

tioning; with the one or the other of these fractions individual immune bodies were always associated. Pick ascertained that the fraction of the horse serum containing no antitoxin was precipitated by 36 per cent. volume saturation of ammonium sulphate solution; the protective portion then came down on the further addition of the precipitant to 44 per cent. Spiro¹ found that the difficultly-soluble globulin (obtained by dialysis) was precipitated by half saturation with potassium acetate. Using the method of Pick, Spiro associated the antirennin of horse serum with the euglobulin, which he considers identical with the half-saturation potassium acetate precipitate. Freund and Joachim² examined yet more closely the precipitation characters of Pick's fractions, finding for both the eu- and the pseudo-globulins soluble and insoluble parts. By the study of the precipitation limits of a number of immune substances, Porges and Spiro³ (without giving any of their experimental work) divide the serumglobulins into three distinct fractions, whose ammonium sulphate precipitation boundaries overlap unless the serum is greatly diluted. As the result of recent criticism, however, the differentiation of several soluble globulins is none too firmly established.⁴

The constant occurrence of the immune substances with the serumglobulin has suggested that these are actually a part of the one or the other of the globulin fractions. An increase in the globulin content of the blood as the result of immunization (Atkinson⁵ and others) is indicative of the serumglobulin nature of these bodies. Joachim,⁶ however, considered he found, in a single observation, that the increase was manifested in the non-protective fraction. Glässner,⁷ in a very recent

¹ Fuld and Spiro, *Zeitschr. f. physiol. Chem.*, xxxi, p. 132, 1900.

² Freund and Joachim, *ibid.*, xxxvi, p. 407, 1902.

³ Porges and Spiro, *Beiträge z. chem. Physiol. u. Path.*, iii, p. 277, 1903.

⁴ The purity of such ammonium sulphate fractions has recently been questioned by Haslam (*Journ. of Physiol.*, xxxii, p. 267, 1905); Osborne and Harris (*Am. Journ. of Physiol.*, xiii, 1905) have emphasized the untrustworthiness of this salt for theoretically differentiating proteids according to their precipitation limits.

⁵ Atkinson, *Journ. of Exper. Med.*, v, p. 47, 1901.

⁶ Joachim, *Arch. f. d. ges. Physiol.*, xciii, p. 558, 1903.

⁷ Glässner, *Zeitschr. f. exp. Path.*, ii, No. 1, 1905.

paper, also states that immunization can be accomplished without any essential globulin change.

Attempts¹ to isolate and establish the non-proteid nature of diphtheria antitoxin and the other immune substances from the standpoint of their digestibility by trypsin have not given satisfactory results. These bodies appear to be very slowly attacked by this enzyme—a character that is possessed in a like degree by the serumglobulins and is independent of the occurrence of an antitrypsin.²

In addition to the globulin separations of the type already discussed,³ methods especially directed towards isolating diphtheria antitoxin have been suggested. These include precipitation along with metallic hydroxides,⁴ combined sodium and potassium chloride separations,⁵ throwing down the antitoxin with zinc salts,⁶ and lastly by precipitating out the non-antitoxic proteids with potassium alum, and subsequently separating the globulins remaining in solution.⁷

The methods which have been proposed for the isolation or concentration of antitoxins, then, are for the most part peculiar and tedious ways by which the "globulins" were finally separated from serum and milk. Evaporation and freezing⁸ have been employed for concentration, but the use of these methods has not been continued. Pick states that by the isolation of his pseudoglobulin or higher ammonium sulphate fraction it is possible to concentrate the protective properties ten to fifteen times. Pick's method is superficially the most practicable. Considerable quantities of antitoxin, however, may be carried

¹ Belfanti and Carbone, *loc. cit.*; Pick, *loc. cit.*; Brieger, *Festschrift für R. Koch*, Jena, 1903.

² Oppenheimer, *Beiträge z. chem. Physiol. u. Path.*, iv, p. 279, 1903.

³ Brieger and Ehrlich, *Zeitschr. f. Hyg.*, xiii, p. 336, 1893; Wassermann, *ibid.*, xviii, p. 236, 1894.

⁴ Aronson, *Berl. klin. Wochenschr.*, 1894, p. 425.

⁵ Brieger and Boer, *Zeitschr. f. Hyg.*, xxi, p. 259, 1896; Astros and Rietsch, *Compt. Rend. Soc. Biol.*, lii, p. 337, 1900.

⁶ Brieger and Boer, *loc. cit.*

⁷ Freund and Sternberg, *Zeitschr. f. Hyg.*, xxxi, p. 429, 1899.

⁸ Bujwid, *Centralbl. f. Bakt.*, xxii, p. 287; Ernst, Coolidge, and Cook, *Journ. Boston Med. Soc.*, ii, p. 166, 1898.

down with the non-protective fraction¹ on third saturation with ammonium sulphate solution (Brieger).

There is little record of the actual experimental administration of purified antitoxic globulins. Park² studied the possibility of eliminating serum rashes by treating a considerable number of cases with an antitoxic globulin prepared by Atkinson. Rashes were still produced. The therapeutic effects were no better than were obtained with ordinary serum, and the use of the separated product was on the whole unsatisfactory.

It is important at this point to emphasize the difference between *concentration* and the *practical concentration* of antitoxins. In the latter case the antitoxic globulins must be so prepared as to be ready for immediate administration; the sterility of the solution must be absolutely insured; the product must preserve its clarity better than ordinary serum, and when administered should cause no more irritation locally. If rashes can be altogether eliminated, or at least lessened, so much the better. Further, the keeping qualities must be unimpaired or improved. The method of concentration must be comparatively simple, certain, and inexpensive.

Narrowed down by the conception of the proteid character of antitoxins, an artificial concentration for the present must consist in a separation of the antitoxic globulins. A concentration of more than three or four times is hardly practicable, for there is a limit to the amount of proteid which can be dissolved and to the viscosity of the fluid which can be sterilized through a Berkefeld filter. Failing to find Pick's fractioning entirely satisfactory, I precipitated the serum with an equal volume of saturated ammonium sulphate solution, filtered and extracted the residue with a saturated solution of sodium chloride. The antitoxic globulin is easily dissolved in the chloride solution in spite of the ammonium sulphate present, the non-soluble proteids (globulins, nucleo-proteids, etc.) sedimenting on standing. After filtering, the sodium chloride solution of the antitoxic

¹ Some experimental observations on this subject showed me that antitoxin in a relatively large amount may be carried down with the lower fraction on third saturation with the sulphate. If the serum has been diluted several times, the precipitation results in no noticeable loss.

² Park, *loc. cit.*

globulins is precipitated by the addition of a half volume of saturated ammonium sulphate solution, or, better still, with acetic acid in the usual manner. The filtered precipitate is pressed dry with paper and dialyzed in parchment. If the acid precipitation has been employed, the globulin solution is neutralized in the course of the first few hours of dialysis, which is continued for from two to three days. Sterilization is accomplished by a double filtration through Berkefeld filters, a quarter to one-half per cent. of sodium chloride being added and a preservative used. The potency of the product is ascertained, it is tested bacteriologically, and is finally injected into animals and actually administered at the Department of Health hospitals before distributing.

The sodium chloride separation here suggested is to be preferred, in my opinion, to a simple precipitation or fractioning with ammonium sulphate. With the additional acid precipitation, almost all the sulphate is removed before dialysis. The resulting dilution is about the same as when the sulphate is employed. The antitoxin is practically all recovered and a concentration of between two and three times the original potency is easily and constantly obtained. The sodium chloride separation is a sharp one, the two groups of proteids showing essentially different physical characters as precipitates. The final product is no more viscous than ordinary serum; it is almost colorless, or tinged with hæmoglobin. When dried down at low temperature a beautifully transparent and entirely soluble scale antitoxin can be obtained. Large quantities of serum can be worked over at comparatively small expense.

Tests show that the artificially concentrated antitoxin, kept in small vials in an ice-box in the usual way, preserves its potency as well as the ordinary antitoxic serum. Therapeutically the results obtained are practically identical with the beneficent effects commonly observed. Local irritation is no more marked and rashes seem to be less frequent and severe when the refined antitoxin is administered. Hundreds of cases have been treated with this product in the Department hospitals, yet no infection for which the antitoxin is responsible has resulted.

The method of separation is possible and practicable largely because of the extreme solubility of the antitoxic globulins and

the remarkable retention of this character when compared with the behavior of other proteids under the same conditions. When precipitated with ammonium sulphate of only a fair degree of purity and when treated with saturated commercial sodium chloride solution, the moist precipitate retains its solubility for weeks. The soluble globulin can be repeatedly precipitated and purified to a high degree. The antitoxic properties follow this soluble globulin at every step and are lost or lessened only by such agents as can considerably modify the character of the proteid.

The highly purified soluble globulin, when practically salt-free, is often precipitated by the addition of distilled water; a trace of sodium chloride present brings the proteid again into solution. Of practical importance, perhaps, is the fact that it is precipitated by the addition of an equal volume of alcohol—a resulting concentration in which sodium chloride is easily soluble. These and other chemical characters of the soluble globulins will be more fully discussed in a subsequent paper.

We are at present engaged in a study of the cause of serum rashes, etc., with preparations of the antitoxic globulins purified to a high degree. Some results already obtained with fairly pure experimental products indicate that the separation of the insoluble globulin does not by any means suffice to eliminate these deleterious effects. An 800-unit antitoxin preparation, fractioned and reprecipitated with ammonium sulphate solution, and subsequently extracted, first with acidified, and then with neutral saturated sodium chloride, dialyzed, and made slightly alkaline, produced a considerable number of severe rashes. It seems possible that the rash production in this instance was associated with some irritation due perhaps to a slightly excessive degree of alkalinity. I have also observed development of rash following the administration of an unneutralized acetic acid preparation of the antitoxic globulin. From the extensive use of ten of the latest routine preparations regarding which clinical reports have been received, a very few mild urticarias only have resulted; in all of these preparations, the reaction (to litmus) has been neutral or but very faintly alkaline. The decrease in the frequency and severity of these effects reported from the Department hospitals has been very encouraging.

A more detailed technical description of the method of concentration which I have introduced into this laboratory follows. It is perhaps unnecessary to emphasize the care which should be exercised in working with any proteid solution which is to be injected for therapeutic purposes. Precautions which are of course second nature to the proteid chemist, may be neglected by assistants or others unfamiliar with this line of work. Serious consequences may follow any mistake.

For concentration, antitoxic serum of almost any grade or quality is serviceable; probably citrate- or other plasma could be used with success. Material of a low grade of protective power (150 to 250 units), old and returned stock, as well as highly potent serum, are all utilizable. As deterioration is probably the result of autolytic processes,¹ just as concentrated preparations can be obtained from the old and returned stock as from the fresh serum.

Ten to fifteen liters of serum are precipitated by the gradual addition with stirring of an equal volume of saturated ammonium sulphate (Merck's pure crystalline, at ten cents per pound). After standing an hour or two the precipitate is collected on large folded papers on ribbed funnels. The precipitates are again dissolved in ten to twelve liters of water, the filter paper being removed by straining through gauze; the resulting solution is reprecipitated with ammonium sulphate solution in a volume equal to that of the water added. The precipitated globulins are once more collected on filters and then treated with twice the original serum volume of saturated sodium chloride solution; the filter paper is strained off as before. The sodium chloride extract is allowed to settle overnight, and the supernatant solution of the antitoxic globulins is siphoned off and filtered. The insoluble residues are again extracted with salt solution, and the washings are combined with the first sodium chloride extract of the succeeding antitoxin preparation.

The sodium chloride extract is completely precipitated now either by the addition of about half its volume of saturated am-

¹ Atkinson noted a decrease in the magnesium sulphate precipitable globulins of old serum the potency of which had deteriorated correspondingly.

monium sulphate solution, or better, by the addition of about 0.25 per cent. of acetic acid. The final precipitate is filtered off. When sufficiently drained, the proteid and the containing papers are dumped on mats of filter paper so that the folded filters are extended into a semicircle, while still holding the somewhat moist, soft, globulin precipitate. These precipitates are pressed out simply by occasionally changing the absorbent paper wrapped about them. When freed mechanically from the bulk of the adherent folded filters, the precipitate is placed in a bag of heavy parchment paper and dialyzed overnight in running water; it is then neutralized if the acid precipitation has been employed. Dialysis is continued in running water for from two to three days longer, toluol or chloroform being added as a preservative.

After filtering the dialyzed solution through paper pulp, it is roughly sterilized through a Berkefeld after a quarter to one-half per cent. of sodium chloride (c. p.) has been added. The anti-toxin globulin solution is again sterilized by a filtration through a second Berkefeld. A preservative is finally used. In filtering the first few cubic centimeters should be discarded or turned into the next preparation.

The protocols of two of the experimental preparations are given below. No attempt was made to make the chemical technique a very careful procedure—*i. e.*, the insoluble globulin precipitates were not again extracted, etc. As it is, they show about four-fifths of the antitoxic property recovered:

PREPARATION XV. 9000 c.c. serum; potency 200; net	
total antitoxin (corrected for two large samples	
taken during preparation).....	1,672,000 units
Final product (500 units per c.c.).....	1,400,000 "

PREPARATION XVI. 9000 c.c. serum; potency 300;	
total	2,700,000 "
When neutralized after 18 hrs. dialysis, 2260 c.c. (900	
units).....	2,358,000 "
Final product, 3320 c.c. 700 units per c.c.	2,224,000 "

It is frequently stated¹ that artificial concentration of anti-toxin is superfluous, because it is possible to immunize animals

¹ Oppenheimer. *Toxine u. Antitoxine*, 1904, p. 87.

to such a high degree that any further procedure is unnecessary. The production of a highly potent serum in any horse, however, is an uncertain process. For this purpose only 25 per cent. of the horses tried by us are serviceable, and even if suitable, the horses can be used for only a few months when the immunization is forced to produce a grade of 500 units per cubic centimeter. An artificial concentration of low-grade serum is possible probably at no more, and perhaps even less, expense than obtains in the present procedure for producing an equally potent antitoxin; considerable saving may also result from utilizing serum of a grade below 200 units, as well as the returned serum, which is at present usually destroyed. A further reduction of expense by working over citrate-plasma instead of serum seems possible. Again there may arise occasions in any serum laboratory when the production of a supply of highly potent antitoxin at short notice is most desirable. For circumstances of this character an available method for artificially concentrating the protective properties of serum might be of considerable service. The elimination of the serum rashes, even in part only, makes the expense question almost a negligible factor in the concentration and refining of antitoxins.

The work so far done and planned is somewhat comprehensive in scope and is both practically and scientifically important. It has to do not solely with the concentration of antisera and the elimination of the serum rashes, etc., but may also throw some light on the chemical characteristics and the nature of antitoxins and related substances, and on the probable functions of the so-called "serumglobulins."

In concluding, I desire to express my thanks to Dr. William H. Park, director of this laboratory, for his active and helpful co-operation.

THE OXIDATION OF AMIDO-ACIDS WITH THE PRODUCTION OF SUBSTANCES OF BIOLOGICAL IMPORTANCE.

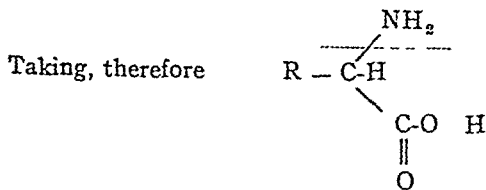
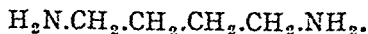
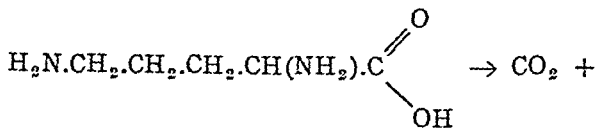
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(From the Laboratory of Dr. C. A. Herter.)

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The mechanism of the various changes which the products of proteid digestion undergo during their degradation in the animal organism is almost wholly unknown. From the standpoint of pure organic chemistry, the transformation of an amido-acid into substances belonging to the carbohydrate group is not at all easy to understand, but yet it cannot be doubted that such changes take place in the organism.

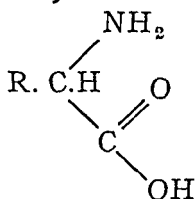
That enzymes are present in the liver capable of removing ammonia from the amido-acids is clear from the work of Lang and others. It is probable too that carbon dioxide is readily removed from the carboxyl group of amido-acids by means of enzyme activity. Thus the author has observed the conversion of ornithin into tetramethylene diamine as a result of digestion with liver tissue, and a similar type of reaction is constantly met with in biochemical changes brought about by living organisms.



representing a typical amido-acid derived from the hydrolysis of proteid, one might expect that in some of the chemical transformations it may undergo, ammonia and carbon dioxide will be removed while a carbon-rich alkyl group is left. The mechanism of the transformation of this group, whether into carbohydrate or even to simple carbon dioxide and water, is completely unknown.

It seemed of interest to know more of the chemical decomposition which the simple amido-acids undergo, and in particular to try to study the products formed by changes whose character approximates as closely as possible to biochemical reactions. The reaction which has first been studied is the oxidation of the amido-acids by means of hydrogen peroxide and a trace of a catalyst such as ferrous sulphate. This method of oxidation is due to Fenton and has led to most beautiful results in the chemistry of the carbohydrate group. A feature of the reaction is that the changes are brought about at ordinary temperatures. Some three years ago I observed that α -amido-isovaleric acid and leucin (α -amido-isobutylacetic acid) on treatment with this reagent are readily decomposed with formation of volatile products. The further study of the reaction has been rendered possible by the kindness of Professor C. A. Herter, to whom I am indebted for the opportunity of making the present communication.

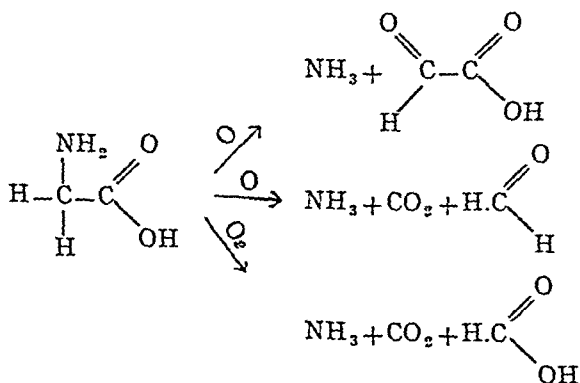
The results at present are incomplete, but some particulars may be given of the oxidation of glycocoll, alanin, and leucin. As is so often the case, the first member of a series offers some differences from its homologues. The three amido-acids mentioned may be represented by the formula,



where R is either a hydrogen atom in glycocoll or a methyl or isobutyl group in alanin or leucin. On oxidation, they all are readily resolved at the *ordinary temperature* into carbon dioxide and ammonia and an aldehyde. The liberation

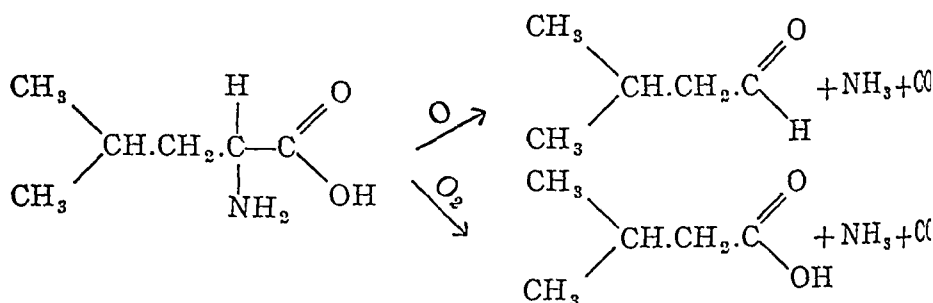
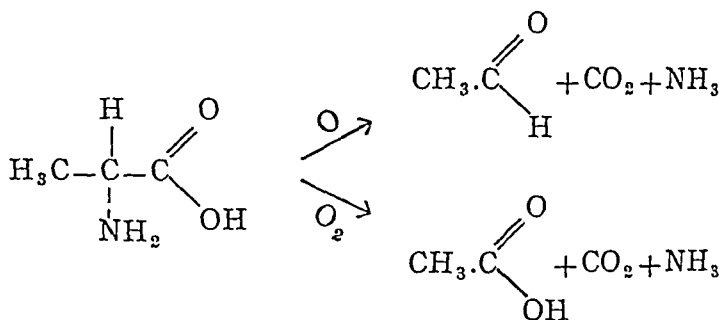
of ammonia and carbon dioxide is completely analogous to their formation in the case of the enzyme reactions previously mentioned, while the extremely reactive aldehydes are obviously substances which could be quickly transformed into other products, as is believed to be the case with formaldehyde in the photosynthetic production of carbohydrate in plants.

In the case of glycocoll, formic aldehyde is produced, and the conversion of this substance into reducing sugars (α -acrose and formose) has already been accomplished in the laboratory by Butlerow and by Fischer, so that it is possible to effect the conversion by purely chemical means and at low temperatures, of glycocoll—a fragment of the proteid molecule—into carbon dioxide, ammonia (or urea), and a carbohydrate. In this connection it is of interest to note that Embden and Salomon have shown that the giving of glycocoll largely increases the output of sugar in diabetic dogs deprived of their pancreas. In the case of the oxidation of glycocoll, formic acid and glyoxylic acid are formed at the same time, and the large production of the latter substance accounts for the small quantity of formaldehyde produced, compared with the yield of aldehydes from alanin and leucin. The formation of glyoxylic acid in itself is not without interest as this substance is known to be commonly present in unripe fruit, and apparently is converted into sugar during the process of ripening. Their mode of origin is obvious from the following scheme:



In similar fashion alanin yields acetaldehyde and acetic acid

(but no pyruvic acid, $\text{CH}_3\text{CO}\cdot\text{COOH}$, the product corresponding to glyoxylic acid), while leucin yields isovaleric aldehyde and isovaleric acid.



The experimental details of a typical oxidation are as follows: 1.0 gram of alanin was added to the calculated amount of hydrogen peroxide solution, which had been standardized by potassium permanganate and also carefully neutralized with sodium carbonate. A few milligrams of ferrous sulphate were added and the whole allowed to stand at the ordinary temperature of the air. After a couple of minutes the solution became slightly warm and a slow evolution of carbon dioxide commenced, while the smell of acetaldehyde steadily increased. After standing overnight the mixture was slowly distilled and the first portion of the distillate caught in a freezing mixture. The distillate smelt strongly of acetaldehyde, and gave all the typical reactions, including the reduction of Tollen's silver solution, the production of aldehyde resin with caustic soda, the iodoform reaction, and also the reaction with sodium nitroprusside in the presence of secondary amines which is characteristic of acetaldehyde as opposed to formaldehyde, ketones, and most other alde-

hydres (*cf.* Beilstein's *Handbuch Ergänzungsband*, i, p. 471). Finally, with phenyl hydrazine a good yield of the β -hydrazone (m. p., 66°C.), ethylidenephénylhydrazine, $\text{C}_6\text{H}_5\cdot\text{NH}\cdot\text{N}:\text{CH}\cdot\text{CH}_3$, was obtained.

The residue, after distilling off the aldehyde, was acidified with dilute sulphuric acid and redistilled. An acid distillate was obtained, while at the same time a small additional quantity of acetaldehyde was liberated, doubtless set free from combinations it had entered into with some of the amido compounds present.

The distillate was examined for pyruvic acid with a negative result, but contained about 0.06 gram of acetic acid. It was identified by the ferric chloride reaction and by conversion into the barium salt.

On analysis the salt gave 53.7 per cent. barium.
Calculated for $(\text{CH}_3\text{COO})_2\text{Ba}$, Ba = 53.8 per cent.

In a similar way the oxidation of glycocoll led to the isolation of formic acid which was converted into lead formate.

Analysis gave 69.5 per cent. metallic lead.
Calculated for $(\text{HCOO})_2\text{Pb}$, Pb = 69.7 per cent.

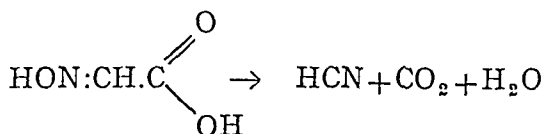
The acid gave also the characteristic reactions with mercuric and silver salts. A large quantity of glyoxylic acid was formed which was identified by means of the phenylhydrazone and by the tryptophan reaction of Hopkins. The quantity of formaldehyde was small, but it was readily detected by the Tollen's aldehyde silver reagent, and by the Lebbin method for the estimation of formaldehyde, and further by the color reactions with concentrated sulphuric acid and resorcin and pyrogallol.

On making alkaline the residue from the distillation of the volatile acids and redistilling, a large quantity of ammonia was liberated and was identified by conversion into the platinum salt. Hydroxylamine was completely absent.

On analysis the salt gave 43.9 per cent. Pt.
Calculated for $\text{Pt}(\text{NH}_4)_2\text{Cl}_6$, Pt = 43.9 per cent.

It is of interest to note that the oxidation of glycocoll is peculiar in that small quantities of nitrogen-containing substances

are produced which are soluble in ether and are probably oximido-acetic acid and formaldoxime. The quantity is, however, very small and their isolation offers peculiar difficulties which need not be entered upon here. Their formation, however, would explain the production of hydrocyanic acid from glycooll by oxidation with nitric acid, observed by Plimmer, since they break up readily with production of hydrocyanic acid.



The direct oxidation of glycooll by peroxide, however, does not yield hydrocyanic acid. An analogous formation of oximes has been observed by Wollfenstein and Haase in the oxidation of piperidin.

Leucin is very readily attacked by the peroxide reagent, and a very vigorous evolution of carbon dioxide and a strong smell of isovaleric aldehyde are immediately obvious. On distillation, isovaleric aldehyde was readily separated and a similar hydrazone to that given by pure isovaleric aldehyde was readily obtained. A small quantity of a volatile substance giving the iodoform reaction was also present and is still under investigation.

On acidifying and re-distilling, isovaleric acid was obtained and identified by conversion into the zinc salt.

The further investigation of the oxidation of proteid decomposition products, including the oxyamido- and diamido-acids is in progress.

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ON THE SOLUBILITY OF URIC ACID IN BLOOD SERUM.

BY ALONZO ENGLEBERT TAYLOR.

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(Received for publication, November 16, 1905.)

The solubility of uric acid in water was determined by His and Paul¹ to be about 1 in 39,500. Uric acid is very slightly dissociated, less than 10 per cent. in saturated solution. His and Paul have, however, confined the solubility within too narrow limits. If one shakes large crystals of uric acid in water for a day at room temperature, one cannot reach a solubility of 1 in 40,000; if, however, one triturates a fine powder of uric acid in water for several hours, one can obtain a solubility of 1 in 35,000. This is simply a repetition of the familiar experience of the physical chemists in the preparation of saturated solutions of calcium sulphate for purposes of standardization. I am acquainted with no published researches in which the solubility of uric acid in the body fluids has been determined with methods of precision. It has been long recognized that in no known disease is the blood serum saturated with uric acid. Even in those specimens of blood from which uric acid will crystallize on the immersion of a thread, the solution is not saturated; this may be easily shown in the simple experiment of adding more uric acid in substance and observing that some of it will be dissolved. The assumed greater solubility of uric acid in blood as compared with water has been generally attributed to the alkaline reaction of the blood. This explanation is without foundation, since the blood is not alkaline in the real sense at all, but practically as neutral as distilled water. With the demonstration of the neutrality of the blood,² a mass of hypotheses on

¹ His and Paul, *Zeitschr. f. physiol. Chem.*, xxxi, p. 1, 1900.

² Fränkel, *Arch. f. d. ges. Physiol.*, xcvi, p. 601, 1903; Fakkas, *ibid.*, xcvi, p. 551, 1903; Höber, *ibid.*, xcix, p. 572, 1903.

gout was obliterated. The blood does, however, contain many different salts, and it is possible that the uric acid anion might displace some weaker anion from one of these salts. About the only known anions that the uric acid anion could be believed to displace are carbonic acid (CO_3^-) and protein. Up to the present we know of no other anions in the blood plasma with affinity constants so low as that of uric acid. That the solubility of uric acid in the blood is much greater than in water, is true; that it circulates in combination with the ordinary cations like sodium, is improbable.

To measure the solubility of uric acid in blood serum the following experiment suffices: Ox serum is filtered and a stream of carbon dioxide passed through it until the first trace of a cloudiness is noted. The gas is now withdrawn and the container tightly stoppered; the trace of precipitation disappears. Such a serum contains more carbon dioxide than normal bovine blood, and has about the same conductivity, but a slightly acid reaction. To this serum pure crystalline uric acid is added in excess, and the container tightly stoppered and shaken in a mechanical shaker for some six hours. It is then allowed to stand overnight, following which the supernatant fluid is drawn off and filtered rapidly through such filter paper as is adapted to the retention of the finest particles, such as barium sulphate. Centrifugation and microscopic examination show these filtrates to be free of particles. Filtration through infusorial filters have yielded identical results. To be entirely certain of the technique it would be advisable to carry out the filtration in an atmosphere of carbon dioxide; control tests have, however, shown the results to be identical if the filtration be rapid. To 500 parts of the filtrate are then added 50 parts of concentrated hydrochloric acid, and the mixture kept at the simmering point for several hours. To the boiling-hot fluid alkali is then carefully added until the protein is precipitated. Some of the protein will have been hydrolyzed, but that is of no consequence to the experiment. The purin bodies in the filtrate, after the removal of the coagulated protein, are then precipitated with cupric sulphate-sodium bisulphite (method of Krüger). The precipitate is collected, carefully washed with hot water, finally suspended in hot water, the solution acidulated, and the copper

precipitated with hydrogen disulphide. When this precipitation is completed (this is a tedious operation, on account of the tendency of the copper sulphide to pass into the colloidal state), the solution is filtered, the somewhat cloudy filtrate evaporated to dryness, the residue taken up in a little hot solution of sodium carbonate, filtered to remove the last traces of copper, the filtrate neutralized, and the purin bodies then precipitated with silver nitrate according to the method of Salkowski. From this point on, the analysis is done according to Salkowski, except that I have controlled the quality of the yield by an estimation of the nitrogen according to the method of Kjeldahl.

Several of the procedures require comment. The serum is boiled with hydrochloric acid, because the uric acid is held in solution in some state that resists recovery by precipitation with copper or silver. If one were to remove the protein by coagulation and then apply the method to the filtrate, one would recover little uric acid. Apparently the boiling with hydrochloric acid splits some combination (reaction of hydrolysis) and sets the uric acid free, following which the metallic precipitations will succeed. Precipitation by cupric sulphate-sodium bisulphite is performed first, because it is under the circumstances more quantitative; the silver nitrate will not effect a complete precipitation without some previous purification of the solvent, which is accomplished by the method of Krüger.

The conductivity of the blood serum was determined by the method of Kohlrausch, employing a standardized decadic rheostat. The reaction was determined by means of the measurement of the electromotive potential, using the Nernst method of concentration cells. The chain was set up as follows: H-1/100 normal HCl in 0.9 per cent. NaCl-0.9 per cent. NaCl-blood-H. In this manner the error of the contact potential was reduced to the minimum. The voltage was measured by the compensation method with the use of the capillary electrometer, and the storage cell checked up before and after each measurement by a standard Weston cell. In the application of this method I employed a modification somewhat different from those used by Fränkel,¹ Fakkas,² and Höber,³

¹ Fränkel, *loc. cit.*

² Fakkas, *loc. cit.*

³ Höber, *loc. cit.*

and designed to prevent the loss of carbon dioxide during the period of measurement. This modification, suggested to me by my colleague, Dr. F. G. Cottrell, Instructor in Physical Chemistry in the University of California, consists in the use of rather shallow cells, with palladium electrodes of such dimensions as to nearly equal the cross-section of the glass cell, and in the sinking of the electrode just below the surface of the blood. When now, the electrodes having been charged equally with hydrogen and known to be balanced by a test with a galvanometer, such an electrode is immersed just beneath the surface of the blood and hydrogen gas passed into the cell above the layer of blood, the electrode soon reaches a constant reading on account of the rapid saturation of the surface layer with the gas, and the carbon dioxide is not dissipated from the blood to any extent. The fellow electrode in the centimormal acid requires of course no such treatment, and when after the completion of a measurement the two electrodes are tested, they will be found to have retained their balance in hydrogen charge. I have compared this apparatus with those of Fränkel, Fakkas, and Höber, and have obtained identical results; I believe it to be more certain than the method of Fraenkel and less complicated than that of Höber. With all these forms of measurement based on the principle of concentration cells, the blood is neutral. The method has a large error in the region of neutrality, an error of probably \pm one power; but with all this allowed for, the conclusion is certain that the blood is neutral if the carbon dioxide be not permitted to escape.

The results of two experiments are given in the table:

Blood.	Specific conductivity. In ohms.	Reaction. CH^+	Uric acid.
(a) 500	109.4×10^{-8}	2.4×10^{-6}	+
(b) 500	104.2×10^{-8}	3.1×10^{-6}	+
After saturation with uric acid.			
(a) 500	106.4×10^{-8}	2.1×10^{-6}	0.546 gm.
(b) 500	104.9×10^{-8}	3.2×10^{-6}	0.489 gm.

It is apparent that the uric acid is soluble in about one thousand parts of blood, *i. e.*, the solution power of blood for uric

acid is some forty times that of water. In this figure are included obviously three magnitudes: (a) the uric acid held in solution in the water of the serum, (b) the uric acid held by adsorption by the colloids, and (c) the uric acid held in some complex combination. Since the serum was acid, we may assume the solubility of the uric acid in the water of the serum to have been the same (surely no greater) as determined by His and Paul.¹ The quantity held by adsorption is not measurable, but it is not inconsiderable, since a solution of neutral globulin in water will hold about five times the amount soluble in the same volume of water. The larger part of the uric acid must therefore be held in some complex combination, in which the uric acid figures in all probability as the molecule.

That the postulated combination of uric acid is in the nature of a complex in which the uric acid figures as the molecule, is indicated by several facts. As is apparent from the table, the conductivity of the blood serum is not altered by the addition of the uric acid, the variations noted are well within the range of error of the method of measurement. The reaction of the blood-serum is not affected by the addition of the uric acid, the variations noted are within the range of error of the method. The reaction of the serum was slightly acid, due to the increased content of carbon dioxide. This was intentional, since otherwise the solution of uric acid might have been ascribed to combinations with cations made available by dissipation of the carbon dioxide. The dissociation of uric acid is very slight, but it is improbable that the quantities of uric acid shown to have been dissolved in the serum could have existed there as such or as simple urates without some variation in one or both of these measurements; when the serum dissolves one part of uric acid in a thousand parts of the solvent without alteration of the conductivity or electromotive potential, one may quite confidently assume that the substance is held in the form of a complex. This is confirmed by the fact that the uric acid cannot be recovered by the commonly effective methods of precipitation. It is known as an experimental fact that when uric acid is mixed with nucleinic acid, thymic acid, or other of the ill-defined nucleinic substances, it resists precipitation in like

¹ His and Paul, *loc. cit.*

manner (Kossel,¹ Minkowski,² Gato³). These experiments are easy of confirmation. Just what may be the nature of the complex in the blood serum in the experiments herein reported, is not known. I have been unable to isolate or define it; it can not be segregated by any method of fractional precipitation known to me.

An obvious possible source of error in the experiment might be sought in the action of the hydrochloric acid on the constituents of the blood serum. These have been eliminated by control tests, which show that the boiling of protein in the manner described leads to no formation of uric acid; and that when uric acid is boiled with hydrochloric acid and a solution of soluble globulin, no such solubility is to be observed.

It will be appropriate here to call attention to the discrepancy between the known facts of the state of uric acid in solution as salts and the numerous and often totally incomprehensible misstatements on the subject to be found in many text-books and in clinical reports. So far as known, uric acid forms with sodium (and with the other cations so far as known) but two salts: the mono-sodium urate— $\text{Na}^+ \text{H}^+ > \text{C}_5\text{H}_2\text{N}_4\text{O}_3^-$, and the di-sodium urate— $\text{Na}^+ \text{Na}^+ > \text{C}_5\text{H}_2\text{N}_4\text{O}_3^-$. The published descriptions of the so-called quadriurate or hemiurate are nothing short of fanciful. I have been unable to find in the literature a physical or chemical description of this alleged salt. I have been totally unable to prepare it, and in this I simply confirm the negative attempts of many other investigators. The di-sodium urate was originally described as neutral, the mono-sodium urate as acid. This misstatement was later partly corrected in the statement that the di-sodium urate was alkaline, the mono-sodium urate neutral. In fact, solutions of these salts in water both present an alkaline reaction, faint in the case of the mono-sodium urate, quite pronounced in the case of the di-sodium urate. Both salts are subject to hydrolytic dissociation, with the production of an alkaline reaction. The studies of His and Paul are beginning to have influence, and we read much less of the relative solu-

¹ Kossel, *Arch. f. Physiol.*, 1893, p. 164; *Zeitschr. f. physiol. Chem.*, xxii, p. 74, 1896.

² Minkowski, in v. Leyden's *Handbuch f. Ernährungstherapie*, ii, p. 499.

³ Gato, *Zeitschr. f. physiol. Chem.*, xxx, p. 473, 1900.

bility of uric acid. The investigations of Fränkel, Fakkas, and Hôber on the reaction of the blood have, however, not yet penetrated into the clinical literature, and we still read of the influence of a varying alkalinity of the blood on the solubility of uric acid. When one reads of the salts of uric acid and the relations of these salts in the body fluids as currently described in many clinical writings, one would be led to infer that cations are to be found lying around free and loose in the body.

The importance of the determination of the solubility of uric acid in the blood to the experimental study of gout is apparent. The question arises at once, does not normal blood contain uric acid? This has been usually denied in the older researches. I have determined uric acid to be present in bovine serum; I have made no study of human serum. Neither have I determined the solution power of human serum for uric acid. The amount of blood required for the analyses will tend to render such investigations unfeasible. It is obvious that the unexpectedly great solubility of uric acid in the blood, and its apparent combination there in the form of some complex, lends foundation to the hypotheses of Minkowski¹ and Schmoll,² which seek to locate in the domain of such complex combinations some of the known relations in gout.

¹ Minkowski, *loc. cit.*

² Schmoll, *Arch. gén. d. Méd.*, lxxxi, 2, p. 2433; *Jour. Am. Med. Assoc.* xliv, 1905.

INVESTIGATIONS ON THE REACTIONS OF INFUSORIA TO CHEMICAL AND OSMOTIC STIMULI.

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California.)

(Received for publication, October 28, 1905.)

I.—THE CHEMOTAXIS OF INFUSORIA IN SOLUTIONS OF ELECTROLYTES.

The various movements and reactions of infusoria have been the subject of a great number of investigations, and it has long been known that when two different solutions are brought in contact there is frequently a reaction of the infusoria in a definite sense,—that is to say, the infusoria are attracted or repelled by one of the solutions. Hitherto we have been unable to anticipate the sense of the reaction, but the experiments which will be described below appear to show that under certain conditions we may, at all events with certain classes of solutions, anticipate the nature of the reaction which will be displayed by the infusoria when two solutions, one of which contains the infusoria, are brought in contact.

In a former paper¹ I developed a theory which aimed at explaining the movements of protoplasm as effects of changes in surface energy in protoplasmic surface-systems—changes which, in most cases, necessarily lead to changes in the distribution of surface and volume, and, therefore, in general, to displacement of the protoplasmic body,² the magnitude and sense of the displacement depending on the sum of mechanical conditions under which the surface-changes take place. These alterations

¹ "An Outline of a Theory of the Genesis of Protoplasmic Motion and Excitation," *Trans. Roy. Soc. of South Australia*, xxix, pp. 7-26, 1905.

² Rhumbler, whose paper I had not read when I wrote my paper on the genesis of protoplasmic motion, has brought forward the same idea to explain amoeboid movements.—*Zeitschr. f. allgem. Phys.*, i, p. 383 and ff. 1902.

in surface-energy were referred to the metabolism of ion-proteid compounds, which leads to the formation or disappearance of electrical double layers, due to free ions, at the protoplasmic surfaces,¹ and therefore to the decrease or increase, respectively, of surface-energy, the sign of the electrical charge on the protoplasmic side of the surface depending on the nature of the preponderating ion-proteid.

One of the special applications of this theory, which were made in the paper to which I have referred, was that to the chemotaxis of amoeboid and ciliated organisms, and the results of twenty-five experiments on the chemotaxis of *Spirostomum*, *Opalina*, and two other species of ciliate infusoria, were entirely in accord with the theory. It was felt, however, that this number of experiments was insufficient to afford undeniable proof of the applicability of the theory to the phenomena of chemotaxis. I therefore proceeded to carry out a more extended series of experiments, the results of which, as will appear in the sequel, are entirely in support of the theory.

The method employed was that used in my former experiments.² A cell of wax on a glass slide was provided with an opening at either end through which capillary tubes containing testing-solutions could be inserted. A small drop of culture thickly populated by infusoria was placed in the cell, and the cell was then filled with a solution and covered with a cover-glass. After a varying period, capillary tubes containing the solutions whose action was to be tested, were inserted through the openings of the cell and supported by the rest of the slide. The advantages of this method are that it enables one readily to compare the actions of two different testing-solutions at once; that one can readily regulate the rate at which the testing-solution diffuses into the cell by simply gumming narrow strips of paper on the slide beneath the capillary tubes; and, finally, that one can obtain a distinct and satisfactory reaction by this method with far fewer infusoria in the cell than by Jennings' method.³ It would, indeed, entail considerable labor to get the infusoria sufficiently concentrated in the medium desired to apply Jennings' method, without introducing so much of the culture as to profoundly modify the "stimulation-efficiency" of the medium. The objection which Jennings

¹ I may point out here that ions liberated by the decomposition of ion-proteid necessarily tend to migrate to the surface as the surface-energy is thereby reduced. See Whetham, *A Treatise on the Theory of Solution*, p. 98, 1902:

² *Trans. Roy. Soc. of South Australia*, xxix, p. 14, 1905.

³ H. S. Jennings, *Journ. of Physiol.*, xxi, p. 264, 1897.

has raised to the capillary-tube method is that some of the infusoria may accidentally stray into, or be stopped by contact with, the tube, and, by evolving CO_2 , attract other infusoria until a clump is formed.¹ This objection does not apply to my method, however, for two tubes are used simultaneously. Ultimately, therefore, in a large number of experiments, the excess of infusoria in or gathered about one tube over those in or about the other tube must be due to the action of the chemical contained in one of the tubes.

The procedure was in every case the same. A small drop of the paramœcium culture (which was an old one) was placed in the cell and the cell filled with an $\frac{N}{50}$ solution of a salt. (This solution is here invariably referred to as the "medium.") Then, after a period varying from three to fifteen minutes, capillary tubes containing the "testing-solutions" (which were also $\frac{N}{50}$ solutions) were introduced and the reactions noted.

The following is a table of the results of one series of experiments in which every possible combination of ten different chemicals was tried. In the left-hand vertical column are the media, and at the top, reading horizontally, are the testing-solutions; + indicates positive chemotaxis; o indicates no reaction; - indicates repulsion or negative chemotaxis; N.O. indicates "not observed"—the medium being too toxic. An asterisk (*) is meant to refer the reader to Part II. of this paper. Thus we read that paramœcia in K_2SO_4 are attracted by HCl , indifferent to Na_2SO_4 and to KCl , and are repelled by NaCl , ZnSO_4 , NaNO_3 , and NaOH , while for the reaction to BaCl_2 and CaCl_2 the reader is referred to Part II. of this paper. A few of the reactions were so slight as to be doubtful; those were the reactions marked o or 2, meaning that there was no reaction or a repulsion, or that there was no reaction or an attraction, respectively. The media, reading downwards, are in the order of their "stimulation-efficiencies." ² The stimulation-efficiency of ZnSO_4 ,

¹ H. S. Jennings, *ibid.*, p. 297.

² In my previous paper I defined the "stimulation-efficiency" of an electrolyte as $\frac{\frac{u}{y_1} - \frac{v}{y_2}}{u + v}$, where u and v are the velocities of the kation and anion respectively, and y_1 and y_2 are the valencies of the kation and anion respectively. This quantity (provided the ratio of the concentrations on the two sides of the protoplasmic surface is approximately constant—a condition which is satisfied when we use equivalent dilute solutions throughout) affords a rough measure of the potential difference at the surface of demarcation between a protoplasmic surface and the medium (*vide* Whetham, *A Treatise on the Theory of Solution*, p. 382, 1902). Since, however, a certain proportion of the ions which enter remains combined to form ion-proteid, and since, besides, an organism cannot be procured in which some ion-proteid is not already present, this quantity must be expected rather to give indications of tendencies than quantitative estimations of the effects of electrolytes (*vide* Robertson, *Trans. Roy. Soc. of So. Australia*, xxix, pp. 11-13).

calculated from the value of the transport-number for dilute solutions,¹ is about $-.14$.

Most of these experiments were repeated two or three times, sometimes four times.

TABLE I.

Medium.	Testing-Solutions.									
	HCl.	K ₂ SO ₄ .	Na ₂ SO ₄ .	KCl.	NaCl.	ZnSO ₄ .	NaNO ₃ .	BaCl ₂ .	CaCl ₂ .	NaOH.
HCl.		N.O.	N. O.	N O.	N. O.	N. O.	N. O.	N. O.	N. O.	N. O.
K ₂ SO ₄ .	+		o	o	—	—	—	*	*	—
Na ₂ SO ₄ .	+	+		o	o	—	—	*	*	—
KCl.	+	+	+		+	o	—	—	—	—
NaCl.	+	+	+	+		—	—	—	—	—
ZnSO ₄ .	N.O.	N. O.	N. O.	N.O.	N. O.		N. O.	N. O.	N. O.	N. O.
NaNO ₃ .	—	—	—	—	o	+		o +	+	+
BaCl ₂ .	—	*	*	—	—	*	o		o +	+
CaCl ₂ .	—	*	*	—	—	*	o	o		+
NaOH.	N.O.	N. O.	N. O.	N.O.	N.O.	N. O.	N. O.	N. O.	N. O.	

* See Part II. of this paper.

In another series of experiments, paramœcia in six different media were tested with HCl and NaOH—all the solutions being $\frac{N}{80}$ solutions as before. The following is a table of the results, the symbols having the same meaning as before. The stimulation-efficiencies are either taken from the table in my former paper, to which I have referred, or are calculated from the value of the transport-number for dilute solutions.²

CuSO₄, ZnCl₂, and MnCl₂ were too toxic in $\frac{N}{80}$ solutions to obtain any chemotactic reactions.

All the chemicals used in these and the preceding experiments were Merck's C. P., except the KNO₃, which was Kahlbaum's, and the MgCl₂, which was manufactured by de Haën.

¹ See Table of Electro-Chemical Properties of Aqueous Solutions in Whetham's *Treatise on the Theory of Solution*, 1902.

² Given in the Table of Electro-Chemical Properties of Aqueous Solutions in Whetham's *Treatise on the Theory of Solution*, 1902.

TABLE II.

Medium.	Stimulation-Efficiency.	Testing-Solutions.	
		HCl	NaOH.
CH ₃ COOK.	+ .352	o *	—
CH ₃ COONa.	+ .114	+ *	—
KNO ₃	+ .015	+	o
NH ₄ Cl.	— .016	—	+
MgSO ₄ .	— .484	—	o
MgCl ₂ .	— .517	—	—

In a third series of experiments, acids and alkalies were used as media and H₂SO₄ and Ca(OH)₂ were used as testing-solutions. The infusorian used in these experiments was colpodium, although some paramœcia were generally present also, and in these cases it was found that the results here tabulated for colpodium apply also to paramœcium. The media and testing-solutions were all used in $\frac{N}{2500}$ concentration, as higher concentrations are too toxic. The following is a table of the results obtained, the stimulation-efficiencies being taken from my former paper.

TABLE III.

Medium.	Stimulation-Efficiency.	Testing-Solutions. ¹	
		H ₂ SO ₄ .	Ca(OH) ₂ .
HCl.	+ .716	o	o
HNO ₃ .	+ .667	+	o
KOH.	— .468	—	+
NaOH	— .604	—	+
Ba(OH) ₂	— .735	—	+

The results are less pronounced and take longer to develop than in many cases where salts are used as media. This, however, may possibly be due to several factors—in the first place, in such dilute media impurities introduced with the organisms may assume considerable importance

¹ The stimulation-efficiencies of H₂SO₄ and Ca(OH)₂ are +.815 and —.758 respectively

in modifying the stimulation-efficiencies of the media, although in these experiments the high velocities of the H^+ and OH^+ ions probably minimize this influence of the impurities. But another factor which is probably of importance in obtaining distinct results is the ratio of the difference between the stimulation-efficiencies of the testing-solution and the medium to the stimulation-efficiency of the medium. Thus, when HCl is used as medium and H_2SO_4 as testing-solution, the difference between the stimulation-efficiencies of the testing-solution and the medium is .099, while when Na_2SO_4 , for example, is used as medium and HCl as testing-solution the difference is .466. Hence, speaking generally, we should expect more pronounced results when salts are used as media and acids and alkalies as testing-solutions than when acids and alkalies are used as media, for the attraction to or repulsion from the testing-solution depends on the *difference* between the stimulation-efficiency of the medium and that of the testing-solution.

On examining Tables I, II, and III, it will be observed that paramoecia or colpodia in a medium which has a positive stimulation-efficiency are attracted by solutions which have a greater positive stimulation-efficiency, are indifferent to solutions the stimulation-efficiencies of which are nearly equal to that of the medium, and are repelled by solutions with a much smaller or a negative stimulation-efficiency. Similarly, paramoecia in a medium which has a negative stimulation-efficiency are attracted by solutions which have a greater negative stimulation-efficiency, and are repelled by solutions with a smaller negative or with a positive stimulation-efficiency, being indifferent only to solutions which have nearly the same stimulation-efficiency. An apparent exception is furnished by their behavior in $NaCl$, which is similar to that in a medium with a positive stimulation-efficiency. This is probably because, the stimulation-efficiency of $NaCl$ being so small, the small amount of culture introduced is sufficient to change its sign.¹ The stimulation-efficiency of the culture itself was positive, for the paramoecia were repeatedly tested in the culture alone and were always attracted by acid and repelled by alkali. Only in one instance did a cluster form in the alkali and that was when the

¹ Possibly, also, chlorides in the medium may decrease the dissociation of the $NaCl$. Thus if the higher velocity of the Cl^+ ion given by recent investigators be correct, this may explain why $NaNO_3$ has not also the sign of its stimulation-efficiency changed by the medium. The $NaCl$ used, however, when tested was found to contain a little potassium.

alkali had been diffusing into the culture for half an hour and had therefore probably changed the sign of its stimulation-efficiency.

The significance of these facts is patent. As I pointed out in my paper on the genesis of protoplasmic motion, 'when a protoplasmic surface-system in a medium of a given stimulation-efficiency is subjected to the influence of a diffusing chemical which has a higher stimulation-efficiency of the same sign, its surfaces tend to increase, owing to increase in the surface charge, while if the diffusing chemical has a lower stimulation-efficiency of the same sign or a stimulation-efficiency of opposite sign, its surfaces tend to decrease, owing to decrease in the surface charge. Hence movement in one sense will take place in the first case and movement in the opposite sense in the second case. Thus, whether we consider that this movement primarily consists in expansion or contraction of the ciliary surface, as Schäfer suggests,¹ and as I assumed in my former paper, or that it primarily consists in contractions of the protoplasm underlying the cilia, as others have suggested, the ciliary beat will be strengthened by all the chemicals which tend to increase protoplasmic surfaces and diminished by those which tend to decrease such surfaces. Thus in the periods of forward motion which occur between the turning movements of paramœcium—motion *towards* the favorable solution will be more rapid than motion *away* from it; similarly, motion towards the unfavorable solution will be slower than that away from it, and the relative rapidity of motion towards and away from a given solution must ultimately be the most important factor in determining the distribution of the infusoria in the cell. We are, however, confronted by the difficulty that in an organism which has one side turned towards a diffusing chemical which leads to increase of protoplasmic surface, the cilia on that side will beat more powerfully and it would appear as if the organism would be turned *away* from the chemical. Ostwald, however, has pointed out that this is by no means necessarily true,² but that the direction of the orientation under such circumstances depends on the relation between the form-resistance

¹ *Anat. Anzeig.*, xxiv, p. 497, 1904.

² *Wo. Ostwald, Pflüger's Arch. f. d. ges. Phys.*, xcv, pp. 49-50, 1903.

("Formwiderstand") of the cilia and the viscosity of the medium on the one hand and the form-resistance of the body of the organism on the other hand. If the resistance to the movement of the cilia offered by the medium in which the organism is swimming be great compared to the resistance to the rotation of the body towards the side on which the cilia beat, then rotation will take place in this sense, and, considering all the cilia replaced by one which has the same effective beat, the body will rotate round a point in this imaginary cilium as centre—that is, the organism will turn *towards* a chemical which leads to increase of protoplasmic surface. The condition of affairs may be roughly imitated by sticking a knitting-needle into the side of some object, such as a cardboard box, which can readily be moved along a smooth table in such a manner that the needle can rotate backwards and forwards about the point of insertion as a centre. If now the point of the needle be rubbed backwards along the surface of the table, the box can be made to slide along and it will rotate in a direction away from the needle. But if the point of the needle be held firmly against the table so that it cannot slide so readily as the box, then if the pressure backwards of the needle against the table be converted into a forwards impulse to the box, the box will rotate around the point of the needle which presses on the table—it will be oriented *towards* the needle. Now it is only necessary to suppose that the form-resistance of the oral surface of paramœcium is greater than the resistance offered to the oral cilia under the conditions of viscosity under which the movements of paramœcia usually take place, while the form-resistance of the aboral surface is less than the resistance encountered by the aboral cilia, to obtain a very easy explanation of the fact that paramœcium always turns towards the aboral side.¹ If the aboral cilia are beating more strongly than the oral cilia, the infusorian will rotate towards the aboral side, because the resistance offered to the rotation of the body towards the cilia is less than the resistance offered to the collective rotation of the aboral cilia towards the body; while if the oral cilia are beating more strongly than the aboral cilia, the organism will

¹ H. S. Jennings, *Journ. of Comp. Neurol. and Psychol.*, xiv, p. 441, 1904.

still rotate towards the aboral side, because the resistance offered to the rotation of the cilia towards the body is less than that offered to the rotation of the body towards the oral side. The form of the oral surface of paramoecium, offering as it does a concavity to the medium, strongly supports such an explanation.

With regard to the experiments so far described, it only remains to mention a few precautions which it was found necessary to take in carrying them out. In the first place, paramoecium is extremely sensitive to differences of osmotic pressure, and it was necessary to use equivalent solutions throughout. Thus paramoecia in $\frac{N}{80}$ KCl are negatively chemotactic towards $\frac{N}{80}$ CaCl₂, but they are positively chemotactic towards $\frac{N}{80}$ CaCl₂. It is also necessary to be careful not to introduce a drop of the testing-solution into the cell with the capillary-tube as it quickly diffuses into the cell and may alter the sign of the stimulation-efficiency of the greater part of the medium. Also, it is necessary to notice whether any infusoria are mechanically pushed up the tube on its insertion, as the reactions of these are now those of infusoria in that testing-solution as a medium. This may occur to infusoria which happen to be immediately in the neighborhood of the mouth of the tube just after its insertion. Such infusoria should always be subtracted from any groups which may form subsequently. In some cases the reaction was very slight (usually when the stimulation-efficiencies of medium and solution were nearly equal). In such cases infusoria which were rotating near the front of the tubes were used as criteria; if the rotatory movements tended on the whole to move the infusoria towards the solution, they were considered to be positively chemotactic toward it; while if they tended to move the infusoria away from the tube, they were considered to be negatively chemotactic towards it. By observing a number of infusoria in this way and repeating the experiment several times a constant result was nearly always obtained.

CONCLUSIONS.

1. So far as I have observed, paramoecia in a medium which has a positive stimulation-efficiency are positively chemotactic to solutions which have a greater positive stimulation-efficiency,

are indifferent to solutions the stimulation-efficiencies of which are nearly equal to that of the medium, and are repelled by solutions with a much smaller positive or a negative stimulation-efficiency. Similarly, paramoecia in a medium which has a negative stimulation-efficiency are attracted by solutions which have a greater negative stimulation-efficiency, are indifferent to solutions which have nearly the same stimulation-efficiency, and are repelled by solutions with a smaller negative or a positive stimulation-efficiency. The apparent exceptions when NaCl is used as a medium are probably to be attributed to the impurities introduced with the culture.

2. Therefore we may reasonably conclude that solutions which tend to increase protoplasmic surfaces attract, while solutions which tend to decrease protoplasmic surfaces repel paramoecia, although the exact mechanism whereby this attraction or repulsion is carried out must be regarded as uncertain until the exact mechanism of the normal swimming movement of paramoecium has been ascertained.

II.—SOME OBSERVATIONS ON OSMOTAXIS¹ IN PARAMOECIA.

The reactions of infusoria to changes in osmotic pressure have been the subject of many investigations. There can be little doubt, and it will be seen still more clearly in the sequel, that many of the phenomena generally classified as chemotactic are really instances of osmotaxis. But when dilute solutions of perfectly dissociated salts are used throughout, and the medium in which the organisms are swimming and the testing-solutions are used in equivalent molecular concentration, the phenomena of attraction or repulsion which are then observed can only be due to chemotaxis, in the true sense of the word. Besides, Jennings has shown that chemotaxis may act in a sense contrary to osmotaxis, so that paramoecia which show positive osmotaxis to distilled water are repelled by a solution of lower osmotic pressure than their medium, if it is a solution of a substance towards which they are negatively chemotactic.² I shall have to refer later to instances of this which occurred during the course of my own experiments.

¹ The term "osmotaxis" is due to Rothert, *Flora*, p. 408, 1901.

² H. S. Jennings, *Journ. of Physiol.*, xxi, pp. 274-284.

The phenomena of osmotaxis are varied and complex. Thus, Massart states that the lower sea-water organisms are in general attracted by solutions of a *higher* concentration than their medium,¹ while paramoecia are, in general, as has been frequently observed, and as we shall see later, attracted by solutions of a *lower* osmotic pressure than their medium. Thus any theory which might attempt to explain the phenomena of osmotaxis would have to take into consideration a number of factors, such as alterations in internal pressure,² coagulation or solution of the proteids, and differences in viscosity.³ But, among the factors which determine the behavior of paramoecium after positive osmotaxis has carried it into a medium of different osmotic pressure, the nature of the ions combined to form its ion-proteids must undoubtedly play a part.

In general, the phenomena accompanying the passage of a paramoecium from a solution of a higher to one of a lower osmotic pressure are as follows: When tested, in a medium consisting of a $\frac{N}{10}$ solution of a salt, with a capillary-tube containing distilled water, they usually exhibit a "motor reaction" on arriving at the mouth of the tube; this does not, as a rule, prevent the organism from entering the tube, and it then usually swims at a moderate rate up the tube to a certain point where it again exhibits a sharp "motor reaction." On passing this latter point the paramoecia exhibit great activity, usually zigzagging up the tube with great velocity with a rapid rotation round the long axis. The precise degree of activity thus displayed varies with the nature of the medium in which they have previously been swimming, but paramoecia from some media show an activity which can only be designated convulsive. After a longer or shorter period the movements become slower and finally normal, and the organisms are now negatively osmotactic to the regions in the tube which are near the medium.

The fact which shows that ionic velocities play a part in the development of these phenomena is that the degree of activity developed after the passage from the medium into distilled

¹ Massart, *Biol. Centralbl.*, xxii, p. 21, 1902.

² Massart, *Arch. de Biol.*, ix, p. 515, 1889.

³ Wo. Ostwald, "Zur Theorie der Richtungsbewegung Schwimmender niederer Organismen," *Pflüger's Arch. f. d. ges. Phys.*, xcv, pp. 46-65, 1903.

water is much greater with media which have high stimulation-efficiencies than with media of low stimulation-efficiency. The reactions to distilled water were tested in the following media, all of them of $\frac{N}{10}$ concentration, namely: KCl, NaCl, NaNO_3 , Na_2SO_4 , K_2SO_4 , BaCl_2 , CaCl_2 , MgCl_2 , MgSO_4 , $\text{CH}_3\text{-COONa}$, CH_3COOK , and NH_4Cl . In all these media the paramoecia were positively osmotactic to the distilled water except in NH_4Cl , NaCl, MgCl_2 , and MgSO_4 , in which no attraction to H_2O was observed. On suddenly dropping paramoecia from NH_4Cl , MgCl_2 , or MgSO_4 into distilled water, however, the usual heightened activity was observed. The activity developed in distilled water was always most marked when the media were solutions of high stimulation-efficiency. Thus the most extreme and exaggerated activity was only shown on transference from the following media: K_2SO_4 , CH_3COOK , Na_2SO_4 , KCl, CaCl_2 , and MgCl_2 , to distilled water.

On transference from a medium of higher to one of lower osmotic pressure the state of affairs which prevailed in the medium is reversed. Instead of the ion with the higher velocity diffusing into the organism more quickly than the ion with the lower velocity, and so giving rise to a difference of potential at the protoplasmic surfaces by decomposition of the ion-proteid formed, the ion with the higher velocity is now diffusing *out* of the organism more quickly than the ion with the lower velocity. Thus, as the ion-proteids undergo katabolism, setting free their ions, we have, *first*, a rapid *decrease* in the potential difference, owing to excessive loss of the ions which have given rise to it, and, *secondly*, a rapid *increase* in the potential difference, owing to more of the slower-moving ions being left behind than of the faster-moving ions. *But the new potential difference is opposite in sign, on the protoplasmic side, to the former potential difference.* Ultimately, as the slower-moving ions diffuse out also, a state of equilibrium sets in, and, in distilled water or solutions of ion-electrolytes, the ions now combined to form ion-proteids in the organism are mainly the hydrions and hydroxyl ions due to the dissociation of water.

Such rapid changes in surface potential-differences in any protoplasmic surface-system must lead to movements, and if we attribute the great activity developed by paramoecia in

passing from a solution of higher to one of lower osmotic pressure to these changes, we can see why it is that the degree of activity is greater when the stimulation-efficiency of the medium is high than when it is low, for the changes are more profound the greater the initial potential difference. Also we can see why the activity is transitory and the movements ultimately become normal, for, as we have pointed out, a condition of equilibrium sets in.

In the first part of this paper I referred the reader to this part for the explanation of certain peculiar results which were obtained in some of the experiments on chemotaxis. These peculiar results were obtained when soluble sulphates were used as media and chlorides of calcium and barium as testing-solutions, or vice-versa, and when acetates were used as media and HCl as a testing-solution. They are tabulated below, the signs employed having the same significance as in the part of the paper just cited:

TABLE IV.

Medium.	Testing-Solution.	Reaction.
K_2SO_4	$BaCl_2$	+
"	$CaCl_2$	+
CH_3COOK	HCl	0
Na_2SO_4	$BaCl_2$	+
"	$CaCl_2$	+
CH_3COONa	HCl	+
$BaCl_2$	K_2SO_4	0
"	Na_2SO_4	-
"	$ZnSO_4$	o
$CaCl_2$	K_2SO_4	-
"	Na_2SO_4	+
"	$ZnSO_4$	+

In all these experiments, except when the medium was CH_3COONa , when the paramœcia entered the tube they showed

exceptional activity, became "negative" to the higher parts of the tube and to the medium, and became slightly swollen—in short, they showed all those phenomena which are usually displayed on transference to distilled water. There can be little doubt that these are all cases of osmotaxis, more or less modified by chemotaxis. That they are not true cases of chemotaxis is shown, for example, by the fact that paramoecia in Na_2SO_4 are "positive" to CaCl_2 , while in CaCl_2 they are "positive" to Na_2SO_4 . We would thus, if this reaction were chemotactic, have the infusoria positively chemotactic to CaCl_2 when it is used as a testing-solution, but negatively chemotactic when used as a medium, which is *a priori* improbable, and, moreover, is contradicted by their behavior in other cases. As Pfeffer has pointed out,¹ when equimolecular solutions are used throughout, in the case of imperfectly dissociated solutions, the *degree* of dissociation must play a considerable part in determining the nature of the reaction. What we have to deal with here are cases of diminished dissociation or even precipitation at the points where the media and testing-solutions come in contact. For example, at the region in the testing-tube where K_2SO_4 or Na_2SO_4 and BaCl_2 meet, a precipitate of BaSO_4 is formed; hence the concentration in this part is diminished and the organisms show positive osmotaxis. In these cases it can be seen that the paramoecia, having once entered it, *are negative to the regions immediately above and below the precipitate*. They do not leave this region. When BaCl_2 is used as a medium and K_2SO_4 or Na_2SO_4 is used as testing-solution the paramoecia are not attracted, because in BaCl_2 they are negatively chemotactic to the KCl and NaCl , which are formed, respectively, when the BaSO_4 is precipitated,² but when ZnSO_4 is used as a medium they are attracted, although ZnCl_2 has a negative stimulation-efficiency. In this case the osmotactic effect masks the chemotactic effect. When CaCl_2 and K_2SO_4 , Na_2SO_4 , or ZnSO_4 meet, no precipitate is formed, but CaSO_4 , which is very slightly dissociated, is formed and diminution in osmotic pressure therefore takes place in these cases also. When CaCl_2 is the medium and K_2SO_4 the testing-solution, however, the chemo-

¹ *Pflanzenphysiologic*, Bd. 2, p. 582, 1904.

² See Table I, this paper.

tactic effect overcomes the osmotactic effect, for in CaCl_2 paramæcia are negatively chemotactic to KCl .¹

When CH_3COOK or CH_3COONa is used as medium and HCl as the testing-solution, acetic acid, which is very slightly dissociated, is formed at the point of meeting, and KCl or NaCl , respectively, is formed at the same time. Here the osmotactic effect would be in the same sense as the theoretical chemotaxis towards HCl were no double decomposition taking place; but the real chemotactic effects in these cases are those due to the KCl and NaCl , respectively. When CH_3COONa is the medium, the osmotactic effect is predominant, though the organisms are too quickly killed by the acid to show increased activity in the region of diminished concentration. When CH_3COOK is the medium, it would appear that the chemotactic effect overcomes the osmotactic. To make sure of this, paramæcia in CH_3COOK were tested with KCl . They were negative to it.

These facts form a remarkable analogy to Loeb's observations on "contact-irritability."² We have seen that infusoria are generally attracted by solutions of lower osmotic pressure than the medium in which they are swimming and that when placed in these new media they are stimulated to great activity; that is, great changes in surface-potentials take place and consequently changes in the areas of the protoplasmic surfaces. These potential changes may either be brought about by distilled water *or by removing an ion from the medium*. Loeb found that by precipitating the calcion (or by removing it through the formation of a slightly dissociated compound, such as CaSO_4) from the superficial layers of muscular tissue the irritability of the muscle was increased so that it contracted on transference from the precipitating solution in which it had been bathed to air or some other indifferent medium. He attributed this to the initial removal of calcions from the superficial layers of the tissue, followed by migration of calcions from the deeper parts to the more superficial parts. It would appear indeed, that the two phenomena—the increased activity of infusoria on removal of an ion and the "contact-irritability"

¹ Vide Table I, this paper.

² *Amer. Journ. of Physiol.*, v, p. 362, 1901.

of muscle—are due to a similar cause. In both cases we have a diminution of surface-potentials, followed by an increase in surface-potentials. To the initial decrease in surface-potentials we may attribute the relaxation which Loeb observed in the contracted muscles when they were again dipped into the solutions which produced “contact-irritability.” The subsequent increase in surface-potential, due to the migration of calcions from the interior of the muscle, would, as usual,¹ cause contractions.

In order to test this analogy still more thoroughly, I used the same solutions for testing-solutions as Loeb used to produce contact-irritability, namely, sodium fluoride, sodium citrate, sodium phosphate, and sodium carbonate, the medium being CaCl_2 , all in $\frac{N}{50}$ solution. Towards the fluoride, citrate, and phosphate of sodium (Na_2HPO_4) they were strongly positively chemotactic, forming thick clusters, especially in the tube containing the phosphate. Towards the Na_2CO_3 , on the contrary, they were negatively chemotactic. A possible explanation of this, however, is the fact that CaCO_3 is soluble in the presence of CO_2 and that, therefore, as the paramoecia are continually evolving CO_2 , no change or very little change in concentration occurs in the fluid immediately surrounding the organism, even in the immediate neighborhood of the tube, while the strong positive stimulation-efficiency of Na_2CO_3 and NaHCO_3 would tend to repel infusoria in CaCl_2 . When in the tubes containing sodium fluoride, citrate, or phosphate, the infusoria did not penetrate the tube beyond a certain height and were then strongly “negative” both to higher and lower parts of the tube. They showed all the phenomena usually observed in paramoecia on transference to distilled water, such as increased activity, slightly swollen condition, etc.

CONCLUSIONS.

1. Paramoecia in a number of media were tested with distilled water. In most cases they were positively chemotactic to the distilled water, but in a few cases the positive chemotaxis was either absent or too slight to be recognized with certainty.

¹ Vide Robertson, *Trans. Roy. Soc. of South Australia*, xxix, p. 29, 1905.

2. The great activity developed by paramœcia on transference from $\frac{x}{10}$ solutions to distilled water is probably to be referred to the ions within the organism which have the higher velocity diffusing out of the organism more quickly than the other ions, and thus causing an initial rapid decrease of the potential differences at the protoplasmic surfaces, followed by a rapid increase in the opposite sense, and this again by a rapid decrease until equilibrium is reached. This explanation is supported by their general behavior on transference to media of lower osmotic pressure, and by the fact that the most extreme and exaggerated activity on transference from any medium to distilled water was only shown when the media had high stimulation-efficiencies.

3. Certain exceptional results obtained in the experiments on chemotaxis are probably due to the interference of osmotactic effects in producing the reaction. These results are in remarkable analogy with Loeb's "contact-irritability" effects. With certain exceptions, the solutions which produce "contact-irritability" also attract paramœcia when the medium is CaCl_2 :

III.—OBSERVATIONS ON THE CHEMOTAXIS AND OSMOTAXIS OF PARAMŒCIA IN SOLUTIONS OF NON-ELECTROLYTES.

At Dr. Loeb's suggestion a series of experiments was undertaken in which solutions of non-electrolytes were used as media, in order to ascertain whether diverse effects on the chemotactic reactions could not be obtained in these as well as in solutions of electrolytes. Since, as regards the formation of ion-proteids, non-electrolytes are indifferent, no such diverse effects are to be expected unless the non-electrolyte has some other effect upon the proteids in the tissue apart from the formation of ion-proteids. It is no part of my theory to exclude the possibility of such effects and the consequent production of diverse reactions in different non-electrolytes, but in cases where such effects do not occur the reaction should be uniform and its sense determined by the small proportion of electrolytes introduced with the culture or by the hydrions and hydroxyl ions of the dissociated part of the water. As will be seen, in the media which I used and in the concentration I used, no diverse effects

were obtained; on the contrary, the reactions were qualitatively the same as they were in the culture alone.

Since $\frac{N}{80}$ solutions of the electrolytes used as testing-solutions are practically completely dissociated, in order to have the media and testing-solutions isotonic it was necessary to use the non-electrolytes in $\frac{N}{80}$ solutions. The following is a table of the results obtained:

TABLE V.

Medium.	Testing-Solutions.	
	$\frac{N}{80}$ HCl.	$\frac{N}{80}$ NaOH.
$\frac{N}{80}$ ethyl alcohol.	Weakly positive.	Negative.
$\frac{N}{80}$ methyl alcohol.	Strongly "	"
$\frac{N}{80}$ cane-sugar.	Weakly "	"
$\frac{N}{80}$ lactose.	" "	"
$\frac{N}{80}$ glucose.	" "	"
$\frac{N}{80}$ mannose.	" "	"
$\frac{N}{80}$ glycerol.	" "	"
$\frac{N}{80}$ urea.	Strongly "	"

Benzene, toluene, xylene, and pyrogallol were too immediately toxic to obtain any chemotactic reaction.

The reactions of the paramœcia in these media towards distilled water were also tested. In all the media they were "positive" to distilled water and showed the usual phenomena displayed by paramœcia on entering the tube containing the H_2O . But in no case was the increase in activity nearly so marked or so exaggerated as it is when the medium is a solution of an electrolyte with a high stimulation-efficiency.

Finally, I desire to express my gratitude to Dr. Loeb for the facilities which he has afforded me for carrying out these experiments and for his valuable advice and suggestions; also to Dr. F. W. Bancroft for his criticisms and advice.

THE QUANTITATIVE ESTIMATION OF THE LECITHANS.

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Among the methods so far devised for the determination of lecithin and related compounds, the one suggested by Hoppe-Seyler,¹ depending on the determination of the ether-soluble phosphorus, has been most commonly used. Like most of the other methods, it does not, however, distinguish between the two main groups of the lecithans, the lecithins and kephalins. Thudichum² has suggested a method depending on the differences in solubility of the lead salts of the various lecithans in alcohol and ether. On account of the complicated manipulations and the amount of material required, it has never come into use. The method of Koch,³ which depends on the determination of the methyl groups split off above and below 240° C. with hydriodic acid, is also rather complicated, but does not require much material.

As very little is known at present of the relative amounts of lecithins and kephalins in the various animal and vegetable substances, an attempt was made to extend the method of Koch to other tissues besides the nervous system. The very first trials with milk (which does not contain much lecithin) gave, however, such unexpectedly high results that we were led to seriously doubt the accuracy of the method. A trial of the reaction with pure butter-fat revealed the fact that fat alone will split off with hydriodic acid either methyl iodide, ethyl iodide, or some other iodide which interferes with the deter-

¹ Hoppe-Seyler, *Handbuch der chemischen Analyse*, 7th edition by Thierfelder, 1903, p. 159.

² Thudichum, *Die chemische Konstitution des Gehirns des Menschen und der Tiere*. F. Pietzcker, Tübingen, 1901, p. 282.

³ Koch, W., *American Journal of Physiology*, x, p. 320, 1904.

mination. This method was therefore abandoned and an attempt made to simplify the method of Thudichum.

The precipitate formed in an alcoholic solution of crude brain lecithin with alcoholic lead acetate has been previously shown by Koch to consist almost wholly of kephalin, as it gives off very nearly all of its methyl groups on heating with hydriodic acid below 240° C. Only traces of methyl iodide are given off above that temperature. A crude preparation of egg lecithin, from which no kephalin can be separated by precipitation with alcohol, also gives a precipitate with lead acetate. As there is a possibility that the precipitate in this case may be a modification of lecithin, it was tested for methyl groups. The result was the same as with the substance from nerve tissues. Only very little methyl iodide could be split off above 240° C. with hydriodic acid. We may assume, therefore, that the lead precipitate always consists of kephalins. As the formation of the precipitate takes place, however, somewhat slowly, it must be hastened by boiling and by the addition of a little ammonia, as described in detail later. The filtrate on standing will still continue to deposit small amounts of precipitate, but the reaction is practically complete and can be used for comparative investigations. The compounds found in the filtrate invariably contain the methyl groups in the proper proportion as required for lecithins. The lecithins are therefore calculated from the amount of phosphorus in the filtrate and the kephalins from the phosphorus content of the precipitate. The lecithins and kephalins before the precipitation with lead acetate are separated from inorganic and extractive phosphates by precipitation with chloroform in acid solution as described later. A careful examination of this precipitate, to be called the lipid precipitate as it contains all the fat-like constituents in the case of all the tissues investigated, revealed the fact that it does not contain any other phosphorus compounds, except in the case of the nervous system. As already mentioned, a sulphur compound which contains phosphorus, not lecithin-phosphorus, is found here and must be corrected for. This necessitates the determination of sulphur in both the filtrate and the precipitate.

For phosphorus determinations the method of Neuman¹ was

¹ Neuman, *Zeitschrift für physiologische Chemie*, xxxvii, p. 131, 1902.

used with some modifications.¹ In the following pages the method of procedure is completely described, even at the risk of some repetition. It has yielded uniformly satisfactory results with the 200 or more analyses carried on in this laboratory during the year.

METHOD OF DETERMINING LECITHINS.

Extraction.

APPARATUS.—A Hopkins' condenser is connected with a flask of 250 c.c. capacity by a glass-ground stopper 40 mm. in diameter. From the return-tube of the condenser a porcelain Gooch crucible of 15 c.c. capacity is suspended by platinum wires. The crucible hangs to within an inch of the bottom of the flask. This apparatus is set up in triplicate and heated by a water or steam bath.

PROCEDURE.—(a) *For solids.*—The material to be analyzed is freed from blood. If necessary, it is ground several times through a mincing machine, and about 10 grams weighed into an Erlenmeyer flask for immediate analysis. If for later analysis the material is put into a glass-stoppered flask and at least 60 c.c. of absolute alcohol added. On starting the analysis, the material is heated just below boiling with 60 c.c. of alcohol for about one-half hour and then transferred to the Gooch crucible, the bottom of which is covered with either filter paper or a layer of asbestos. The filtrate is allowed to drain into the 250 c.c. flask belonging to the extraction apparatus, and the tissues extracted for eight hours in the extractor. If necessary, more alcohol must be added. The alcohol in the flask is then gently evaporated and ether added. The extraction with ether is continued for eight hours. The residue in the Gooch crucible is then removed, ground up in a mortar, transferred again to the Gooch crucible, and extracted six hours with alcohol and four hours with ether. The residue left on evaporating the alcohol and ether is treated as described later.

(b) *For Liquids and Plant Substances.*—The material is weighed and alcohol added as described previously. In the case of large

¹ Hartwell, B. L., Bosworth, A. W., Kellogg, J. W., *Journal of the American Chemical Society*, xxvii, p. 3, 1905.

quantities of liquid, such as milk, the first filtrate is better evaporated in an open dish. The residue insoluble in alcohol is then extracted as described for solids. As the residue from the alcohol-ether solution, in the case of liquids like milk, and also in the case of a large number of plant substances, contains considerable phosphorus (not lecithin-phosphorus), it is transferred to the Gooch crucible and again extracted, but only with ether. The other phosphorus compounds originally soluble in alcohol do not dissolve in ether to any extent. The residue insoluble in ether but soluble in alcohol in the case of plant substances such as corn-meal is often very sticky and difficult to manage, so that great care must be taken to see that it is completely extracted.

EMULSIFICATION AND PRECIPITATION OF LECITHANS.—The last traces of alcohol or ether are removed by gentle heating from the alcohol-ether residue which has been extracted by the alternate alcohol and ether extractions described above. Without removing the residue from the extraction flask, about 40 c.c. of distilled water are added and allowed to stand not longer than twenty-four hours on account of the danger of bacterial decomposition. The material, after this preliminary softening, emulsifies readily and is transferred to a graduated 100 c.c. flask with as little additional water as possible. In case much fat is present, this can be removed from the sides of the flask only with some difficulty. The addition of 1-2 c.c. of chloroform and thorough shaking serves to overcome this difficulty. Gentle scraping with a glass rod, the end of which is covered with a piece of rubber, is also an aid. By the time everything has been removed from the extraction flask there is usually about 90 c.c. of liquid in the graduated flask. This is thoroughly shaken up, 1-2 c.c. of concentrated hydrochloric acid and 2-4 c.c. of chloroform added, the whole well shaken and made up to the 100 c.c. graduation. The amount of chloroform necessary to produce complete precipitation increases with the amount of fat present. If too much chloroform has been added, however, it settles to the bottom in a liquid form and interferes later with the decantation. When much fat is present this is difficult to avoid; but when little fat is present the precipitate clumps together and can be easily washed. The amount of acid must be kept as low as

possible as there is always some danger of hydrolysis. With such tissues as muscle very rich in fat, the addition of at least 2-3 c.c. of acid is absolutely necessary for complete clearing of the supernatant liquid. The danger of hydrolysis of lecithin, although present, is rather slight, as investigated by Coriat¹ and confirmed by one of us.² The settling may take from one day to two weeks, but should not extend any longer. If all the alcohol has not been removed from the residue, the time of complete precipitation will be longer. The *precipitate* will hereafter be designated as the *lipoid* precipitate, as it contains all the fats and fat-like substances such as cholesterin and cerebrin. In the case of nerve tissues, it also contains the sulphur compound probably in combination with kephalin. As the sulphur compound seems to contain phosphorus, independent of its kephalin combination, this must be corrected for. The clear solution above the lipoid precipitate contains all the water-soluble extractives, inorganic phosphates, phosphorus in simple organic combination, and inorganic salts. Thus the lecithans are obtained practically free from all other phosphorus compounds (except in the case of nerve tissues explained above). The precipitate is washed with a solution of 1 per cent. hydrochloric acid to remove all soluble phosphates which adhere mechanically.

SEPARATION OF KEPHALINS.—The lipoid precipitate having settled completely, the clear supernatant liquid is carefully decanted off through a 6-8 cm. ashless filter paper, and the precipitate washed by shaking in the flask with 10 c.c. of water containing 1 per cent. by volume of strong hydrochloric acid. The precipitate settles again in a few minutes and the wash fluid is also decanted through the filter. The precipitate in the flask is dissolved in hot alcohol, the solution transferred to a clean 300-500 c.c. long-necked Jena flask, the glass-stoppered flask, and the filter paper, through which the dilute acid solution had been originally filtered, thoroughly washed with successive portions of hot alcohol, and finally rinsed with a small portion of ether to dissolve the last traces of the kephalin. The volume of the solution is made up to 100 c.c. with alcohol, the solution

¹ Coriat, *American Journal of Physiology*, xii, p. 361, 1904.

² Koch, *Ibid.*, xi, p. 318, 1904.

heated on the water-bath to remove the small amount of ether added, and 5 c.c. of a hot saturated alcoholic solution of lead acetate added to the rapidly whirling solution. The flask is again placed on the water-bath for about ten minutes, 1 c.c. of 50 per cent. ammonium hydrate solution added, the whole vigorously shaken, and allowed to remain on the water-bath about five minutes longer. The flask is then set aside to cool. After a definite time, which must be constant for comparative work (twenty-four hours), the clear supernatant liquid is decanted through as small an ashless filter paper as is practical for the case in hand (usually 6-8 cm.) into a 300-500 c.c. long-necked Jena flask, and the precipitate washed with hot alcohol, and the alcohol washings combined with the filtrate. The precipitate while still on the filter paper is placed over the flask containing the main part of the lead precipitate, a hole punched in the bottom of the filter, and the portion of the precipitate on it completely washed into the flask, using as little hot water as possible. This water is carefully evaporated off over a free flame without charring the organic matter in the flask. In this manner the necessity of burning the filter paper is eliminated. The solution of lecithins in the other flask is evaporated to dryness on the water-bath, the flask being turned on its side as much as possible in order to allow the vapors to flow out through the neck readily.

OXIDATION AS DESCRIBED BY NEUMAN.¹

PROCESS.—To each of the flasks containing the lecithin and kephalin residue respectively, 10 or 15 c.c. (not more) of a mixture of sulphuric acid, sp. gr., 1.84, with an equal volume of nitric acid, sp. gr., 1.42, is added. The flask is placed on the gauze with the funnel-stem extending barely into its mouth. It is then very carefully and slowly warmed with a Bunsen burner. If sufficient care be not taken at first, the reaction will become too violent and the flask may crack because of the too rapid heating. When the brown fumes have cleared away and the liquid has become only slightly colored from charring, the flame is removed, fuming nitric acid, sp. gr., 1.5, very carefully

¹ Neuman, *Zeitschrift für physiologische Chemie*, xliii, p. 32, 1904.

added by drops, and the process continued until the organic matter is completely destroyed, as indicated by the failure of the clear, colorless, or bright yellow solution to become dark as the result of the charring of the organic matter when heated sufficiently high to cause the evolution of white fumes. About twenty minutes is usually required to carry out an oxidation, the time varying with the amount of organic matter to be destroyed. The advantage of using the fuming nitric acid and not the acid mixture lies in the fact that the amount of sulphuric acid is under control and is not added in such excess as to interfere later with the molybdate precipitation.

Estimation of Phosphorus.

Reagents used:

1. Dilute sulphuric acid, made by adding 100 c.c. sulphuric, sp. gr., 1.84, to two litres of water.
2. Ammonium hydrate solution, sp. gr., 0.90.
3. Nitric acid, sp. gr., 1.42.
4. Crystalline ammonium nitrate, or a 60 per cent. solution.
5. Molybdate solution, made according to the formula of Olsen, by dissolving 75 grams of crystalline ammonium molybdate in 500 c.c. of water, and pouring this solution into dilute nitric acid (250 c.c. nitric acid, sp. gr., 1.42, plus 250 c.c. water) in a bottle or beaker, with vigorous shaking or stirring. This is kept in a warm place (65° C.) for several days, until a portion heated to 70° C. gives no precipitate. The solution obtained is filtered or decanted into a glass-stoppered bottle.
6. Ammonium nitrate solution, 0.1 per cent.
7. Phenol-phthalein solution, made by dissolving one gram of the solid in 100 c.c. of alcohol.
8. Half-normal solution of sodium hydroxide, standardized by titrating against half-normal oxalic acid containing 31.51 grams of Kahlbaum's special oxalic acid per litre, and against the half-normal solution of sulphuric acid.
9. Half-normal solution of sulphuric acid standardized by precipitating as barium sulphate and weighing the ignited precipitate obtained.

PROCEDURE.—When cool, the solution obtained by oxidation of a lecithin residue or a kephalin precipitate in the manner described is diluted by the careful addition of 50 c.c. of water, filtered under pressure to remove the lead sulphate, formed by the reaction of the lead salt in the residues with sulphuric acid, and the flask, lead sulphate, and filter carefully washed free from phosphoric acid, using as little of the dilute sulphuric acid

(one volume of acid to twenty volumes of water) as possible, and combining the wash water with the main filtrate. The filtrate is carefully transferred from the filter flask to a 300-500 c.c. flask, rinsing the filter flask several times with a little distilled water.

The filtered solution in the flask is neutralized with ammonia water, sp. gr., 0.90, and acidified with strong nitric acid, adding about 1 c.c. in excess. To this is added about 50 grams of dry ammonium nitrate, or a volume of the 60 per cent. solution containing that amount (65 c.c.), and the volume made up to 200-225 c.c. After heating to 70°-75° C. on the water-bath, 25 c.c. of freshly filtered molybdic solution are added, the flask well shaken, replaced on the water-bath, and kept at 60°-65° C. for six hours. Removing the flask from the water-bath, the solution over the yellow precipitate is decanted, and filtered under pressure through an ashless filter paper supported by a cone of hardened filter paper. The precipitate, flask, and filter are now washed with successive 10-20 c.c. portions of the 0.1 per cent solution of ammonium nitrate¹ until freed from acid, as indicated by the reaction towards phenol-phthalein of the last few drops from the funnel. After complete washing, the filter, with the portion of the precipitate on it, is placed in the flask containing the other portion of the precipitate, about 150 c.c. of water added, and the flask shaken to unfold the filter paper and distribute the precipitate more loosely. Half-normal sodium hydroxide solution is now added from a burette until all the precipitate dissolves on shaking, 4-5 c.c. in excess is added, and the solution carefully heated over a free flame protected by gauze, and boiled until all the ammonia is driven off, as indicated by the reaction of the vapors to litmus paper. This requires about fifteen minutes or more. The flask is then allowed to stand until cool, or cooled under a stream of cold water, 6-8 drops of the phenol-phthalein solution added, and the excess of sodium hydrate titrated with half-normal sulphuric acid. The total number of cubic centimetres of the sodium hydroxide solution added, minus the number of cubic

¹ This method of washing was devised by Dr. Robert M. Bird of the Missouri Agricultural Experiment Station, who has kindly permitted us to publish it here for the first time.

centimetres of sulphuric acid added, gives the number of cubic centimetres of half-normal sodium hydrate required to dissolve the precipitate. This, multiplied by 0.553, the equivalent of 1 c.c. of half-normal sodium hydrate solution in terms of phosphorus according to Neuman, gives the amount of phosphorus found. This, multiplied by the factor for lecithin or kephalin, 25.75, assuming the molecular weight at approximately 800, gives the amount of lecithin or kephalin found.

The results of a number of analyses carried out with this method are given in the following table. It is interesting to note the very common occurrence of kephalins sometimes in larger quantity than the lecithins.

Substance Analyzed.	Lecithins. Per cent.	Kephalins. Per cent.	Total Lecithans. Per cent.
Muscle (masseter)	0.75	0.55	1.30
" "	0.70	0.58	1.28
" (quadratus lumborum)	0.48	1.1	1.58
" "	0.40	1.0	1.40
" (heart)	0.61	1.0	1.61
" "	0.59	1.1	1.69
Gland (submaxillary)	0.97	0.86	1.83
" "	1.0	0.71	1.71
" (pancreas)	0.59	1.3	1.89
" "	0.52	1.2	1.74
Testicle (very young)	1.35	1.43	2.78
" (young)	1.0	1.2	2.2
" (old)	0.67	0.90	1.57
Lung	1.2	1.0	2.2
" "	1.0	1.0	2.0
Kidney (cortex)	1.2	1.4	2.6
" "	1.3	1.4	2.7
Liver	0.8	1.5	2.3
" "	1.5	1.3	2.8
White of egg	0.1	0.1	0.2
Yolk of egg	3.0	6.9	9.9
A. G. F. A. Lecithin	33.0	66.0	99.0
Milk (cow's)	0.049	0.037	0.086
" "	0.036	0.045	0.081
" "	0.045	0.027	0.072
" (human)	0.041	0.037	0.078
Bread	0.12	0.14	0.26

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THE ANALYTICAL METHODS OF SERUM PATHOLOGY.¹

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(Received for publication, November 10, 1905.)

As part of work testing the application of physical chemistry to immunity, measurements were made of the amount of amboceptor present in heated, hemolytic serum after contact with corpuscles, the object being to determine in this way the law governing the absorption of hemolytic amboceptor. The unexpected phenomenon was observed that when serum and corpuscles² were put together in certain proportions, subsequent analysis occasionally showed what was apparently more amboceptor than originally present, in spite of the fact that amboceptor had been removed from the liquid by absorption by the corpuscles. This paradoxical result indicates, either that the interaction of amboceptor and corpuscles is a much more complex phenomenon than heretofore supposed, or that the method of analysis is wrong.

The analysis was performed by the method commonly used in such experiments. Accurately measured quantities of the liquid to be analyzed were allowed to act on corpuscles in the presence of a constant amount of complement, and the resulting hemolysis compared with hemolysis by known amounts of amboceptor, acting under identical conditions. The hemolysis, by the known quantities of amboceptor, was plotted as a curve (AN, Fig. 1,) showing the relation between and amount of hemolysis amount of amboceptor, and the hemolysis by known volumes of the liquid to be analyzed compared directly with readings on this curve.

¹ Presented before the Chicago Pathological Society, June 12, 1905. The cuts used in this paper have been furnished by the Bulletin of that Society.

² *Journal of Infectious Diseases*, ii, p. 460.

The curve so obtained stands for a physico-chemical law, the law governing the liberation of hemoglobin by amboceptor.

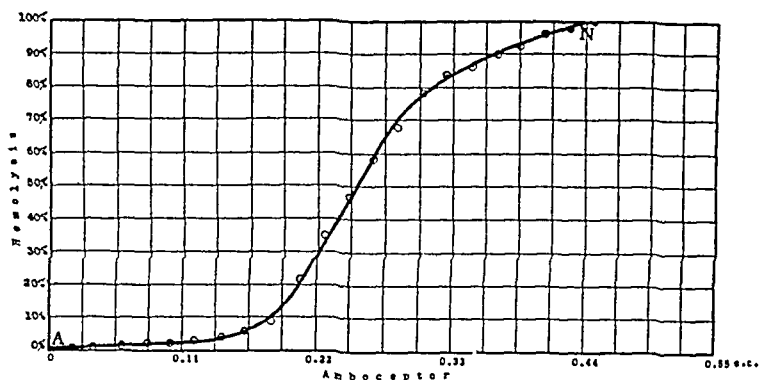


Fig. 1.—THE AMBOCEPTOR CURVE

Corpuscles exposed to increasing amounts of heated, hemolytic serum (amboceptor) in the presence of a constant amount of normal serum (complement). Hemolysis estimated colorometrically as percentages of total lysis.

Any fluid to be analyzable must liberate hemoglobin according to the same physico-chemical law; otherwise duplicate analyses will not agree. If, for example, the new fluid should liberate hemoglobin under a law expressed graphically by a straight

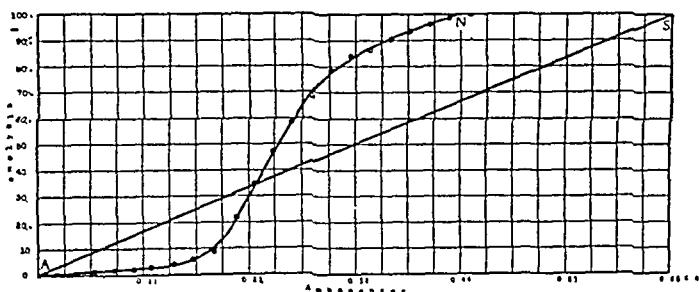
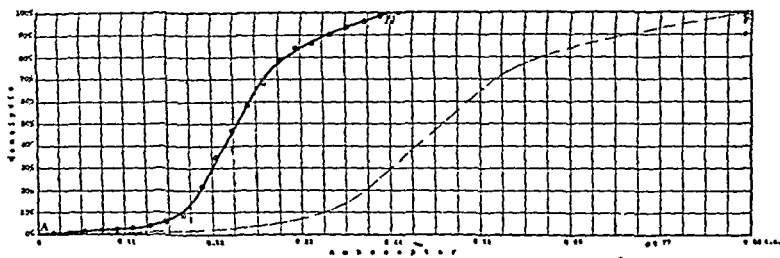


Fig. 2.—CURVES REPRESENTING DIFFERENT PHYSICO-CHEMICAL LAWS.

AN=normal amboceptor curve, AS=hypothetical curve. Sera corresponding to these curves could not be compared quantitatively.

line (AS, Fig. 2), hemolysis with certain volumes would indicate that it contained more amboceptor than the original serum,

while other volumes would give the same or less amboceptor. No conclusion could be drawn from such comparisons.



but 25 per cent. of its original amount of amboceptor; AC that it contains but $28\frac{4}{7}$ per cent.; AD, $33\frac{1}{3}$ per cent.; AE, 40 per

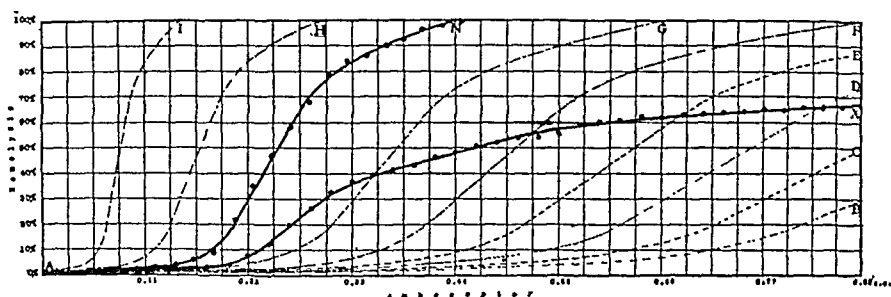


Fig. 5.—AMBOCEPTOR CURVES BEFORE AND AFTER EXPOSURE TO CORPUSCLES.

AN=normal amboceptor curve, AX=curve after exposure to corpuscles. Dotted curves as in Fig. 4. The two curves represent different physico-chemical laws. Sera not directly comparable.

cent.; AF, 50 per cent.; AG, $66\frac{2}{3}$ per cent.; AH, 150 per cent.; and AI, 300 per cent. Any fluid to give a constant result with duplicate analyses must liberate hemoglobin according to a curve similar to one of these.

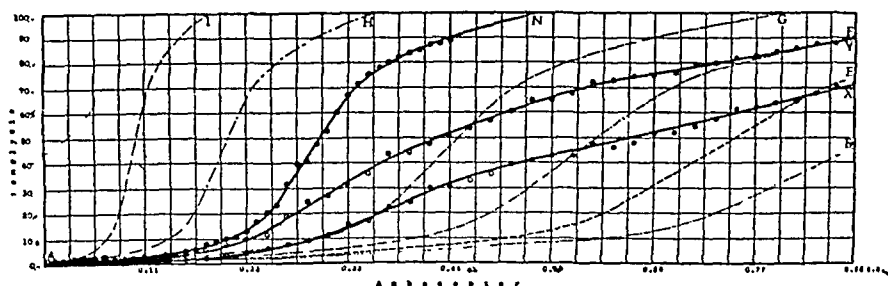


Fig. 6.—AMBOCEPTOR CURVES BEFORE AND AFTER EXPOSURE TO CORPUSCLES.

AN=normal amboceptor curve, AX and AY=curves after exposure to corpuscles. AX differs from AY in that it was made with a larger number of corpuscles. Dotted curves as in Fig. 4. Sera not directly comparable.

Testing serum that has been exposed to corpuscles, a quite different curve is obtained (AX, Fig. 5). This curve not only does not coincide with one of the theoretical curves, but actually

crosses a number of them, so that widely different measurements are possible. In large volumes the fluid would apparently contain less than a third the original amount of amboceptor; in smaller volumes it would apparently contain 40 per cent., 50 per cent., and even over 66 $\frac{2}{3}$ per cent.

Two additional curves are shown in Fig. 6 (AX and AY). These differ from each other only in the fact that in the lower one (AX) the serum was exposed to a larger number of corpuscles. Duplicate analyses drawn from these curves are recorded in the following table.

ANALYTICAL DATA FROM FIGURE 6.

	Volume Analyzed	Apparent Amboceptor Found	Percentage
AX.....	0.88 c.c.	0.343 c.c.	39 Per Cent.
	0.77 "	0.323 "	42 "
	0.66 "	0.303 "	46 "
	0.55 "	0.288 "	53 "
	0.44 "	0.266 "	61 "
	0.33 "	0.224 "	68 "
AY.....	0.88 "	0.440 "	50 "
	0.77 "	0.391 "	51 "
	0.66 "	0.356 "	54 "
	0.55 "	0.330 "	60 "
	0.44 "	0.303 "	69 "
	0.33 "	0.268 "	81 "
	0.22 "	0.198 "	90 "

The impossibility of direct analysis is even more strikingly shown in Fig. 7. Here the original curve and the curve after exposure to corpuscles intersect, so that analysis would show, when small volumes were used, apparently more amboceptor than originally present, while larger volumes would give the same or less amboceptor.

Examining work in other fields of immunity, one is struck with the numerous possibilities of similar sources of error. A toxin-antitoxin mixture is compared directly with pure toxin, filtered and non-filtered sera are considered as acting according

to the same quantitative laws, far-reaching conclusions are drawn from measurements of the agglutinating power before and after exposure to bacteria, and complex formulæ are de-

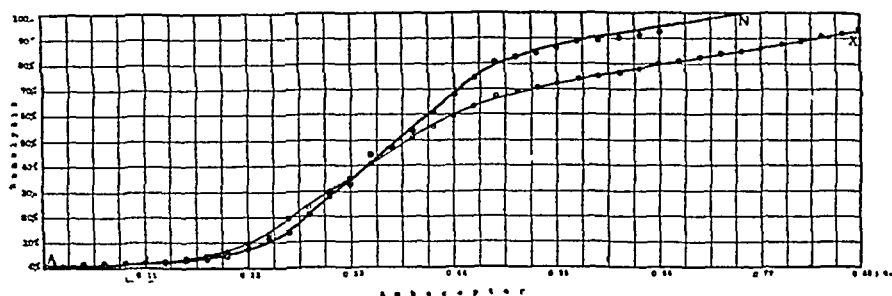


Fig. 7.—INTERSECTING AMBOCEPTOR CURVES.

AN=normal amboceptor curve, AX=curve after exposure to a minimal number of corpuscles. These curves explain certain apparently paradoxical results.

duced from actions at different temperatures. Throughout serum-pathology there is the assumption that numerous dissimilar fluids are analytically comparable. In most cases this assumption rests on no experimental basis. A re-examination of a large part of the quantitative work thus far done is, therefore, necessary to rule out a possible source of error.

A METHOD FOR THE DETERMINATION OF HYDROGEN PEROXIDE IN MILK, TOGETHER WITH SOME OBSERVATIONS ON THE PRESERVATION OF MILK BY THIS SUBSTANCE.

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(Received for publication, October 16, 1905.)

In 1893, Richardson,¹ in a paper which has not received the attention it merits, pointed out that urine when exposed to sunlight and air, does not undergo putrefactive decomposition. He proved that hydrogen peroxide was formed under these conditions in quantities sufficient to prevent bacterial growth. Richardson introduced a new method for the quantitative estimation of hydrogen peroxide based on the yellow color given by it with titanium hydrate dissolved in sulphuric acid. This yellow color is due to the formation of titanium peroxide and was estimated by comparison with a standard solution of this substance. I have found that this reaction lends itself readily to the detection of hydrogen peroxide in milk. On the addition of a titanous acid solution (titanium hydrate dissolved in sulphuric acid) to a few cubic centimeters of milk containing hydrogen peroxide, a coloration appears, which varies between a light yellow and a deep orange according to the amount of hydrogen peroxide present. Lévy² described a number of color reactions given by titanous acid with different phenols, and it was found that the addition of titanous acid to milk containing salicylic acid gives a color reaction which can scarcely be distinguished from that given by hydrogen peroxide. In applying the test to milk this fact must be borne in mind. But it is not difficult to determine by means of other tests whether the reaction is due to the presence of salicylic acid or to hydrogen

¹ Richardson, *Journal of the Chemical Society*, lxiii, p. 1109, 1893.

² Lévy, *Annales de chimie et de physique*, xxv, p. 433, 1892.

peroxide. Besides showing qualitatively the presence of hydrogen peroxide in milk, the titanous acid reaction may be used to determine its amount with approximate accuracy. The method can be best illustrated by an example. The milk used was always boiled to prevent catalysis of the hydrogen peroxide. Commercial hydrogen peroxide was neutralized with barium hydroxide and distilled under a pressure of about 50 mm. Its strength was determined by titration with potassium permanganate. A standard solution of titanium peroxide was prepared in the following manner: titanium dioxide was boiled with concentrated sulphuric acid, and after cooling it was diluted with water and filtered. The filtrate was treated with ammonium hydrate and the precipitate of titanium hydrate was separated by filtration, carefully washed, and dried. The dry powder was dissolved in 25 per cent. sulphuric acid and treated with an excess of hydrogen peroxide. This solution of titanium peroxide was diluted with sulphuric acid and its strength determined by placing a known amount of hydrogen peroxide in a Nessler tube of 50 c.c. capacity. To this an excess of titanous acid was added and the tube filled up to the mark with dilute sulphuric acid. The titanium peroxide solution was added from a burette to a second Nessler tube containing dilute sulphuric acid, until the color of the second tube matched that of the first. The average of several concordant determinations was taken. A specimen of boiled milk was so prepared that it contained 0.00096 gram of hydrogen peroxide in 25 c.c. Of this milk, 5.0 c.c. were diluted with an equal volume of water, and then an excess of titanous acid was added (5.0 c.c. of a 1.5-2.0 per cent. titanium hydrate solution in 25 per cent. sulphuric acid). This mixture was filtered first through cheese-cloth and then through paper pulp aided by gentle suction, the precipitate in each case being washed with dilute sulphuric acid. This was found to be the quickest method of obtaining a filtrate sufficiently clear for colorimetric comparisons. The filtrate was placed in a Nessler tube, which was then filled up to the mark with dilute sulphuric acid. This was compared with the previously determined titanium peroxide solution described above. Even in the absence of hydrogen peroxide a discoloration in the milk filtrate is seen after

treatment with the titanous acid reagent. This is due to the action of the sulphuric acid in the reagent on the milk, and it would be a source of considerable error if left uncorrected. The difficulty was overcome by adding to the standard titanium peroxide solution an equal amount of a filtrate prepared from the same specimen of milk exactly as above indicated, except that 25 per cent. sulphuric acid was used instead of the titanous acid reagent. The color produced by sulphuric acid varies with different specimens of milk, and therefore this filtrate must be prepared from the milk which is being tested. As already stated, the standard solution of titanium peroxide contained an excess of hydrogen peroxide, and hence the presence or absence of hydrogen peroxide in the milk filtrate which is added to the standard solution is of no consequence.

The tube containing this blank milk filtrate was filled up nearly to the mark with dilute sulphuric acid and the standard titanium peroxide was added from a burette until its color exactly matched that of the first tube.

One c.c. of the standard solution corresponded to 0.000094 gram of hydrogen peroxide. In the case given, 5.0 c.c. of milk required (a) 1.9, (b) 1.85 c.c. of the standard solution. According to this, the 25.0 c.c. would contain 0.00088 gram of hydrogen peroxide while actually 0.00096 gram was added. In the following table the results of some of the experiments are given:

No.	Milk + H_2O_2 , c.c.	H_2O_2 added, Grams.	H_2O_2 found, Grams.	H_2O_2 added, Per cent.	H_2O_2 found, Per cent.	Per cent. of H_2O_2 , recovered.
(1)	25.0	0.00050555	0.000425	0.0020222	0.0017	84.0
(2)	25.0	0.00096	0.00088	0.00384	0.00352	91.7
(3)	5.3	0.0003114	0.000285	0.005876	0.00538	91.5
(4)	25.0	0.0020336	0.0019.	0.008134	0.007687	94.4
(5)	5.5	0.0005184	0.0004977	0.0094	0.009	95.7
(6)	5.5	0.0005184	0.0004932	0.0094	0.00899	95.6
(7)	25.5	0.0039504	0.002796	0.0122	0.0112	91.7
(8)	67.0	0.0098496	0.0094867	0.0147	0.0141	95.9

The amount of hydrogen peroxide in (1) was not sufficient to permit of a very good colorimetric comparison, and this amount, therefore, seems to mark the limit of our method. This limit may vary somewhat with different specimens of milk on account

of the more or less intense color of the milk filtrate. It may be mentioned that the addition of titanous acid to milk containing such small amounts of H_2O_2 hardly gives an appreciable color. The coloration only becomes distinct after filtration. From the table it is seen that the limit of an approximately accurate determination of hydrogen peroxide in milk lies between (1) and (2). Since (2) with about 0.0002 gram of hydrogen peroxide gave a fairly accurate result, the limit of the quantitative method is about 1:5000.

In some of the first experiments the milk containing the hydrogen peroxide had been standing for some time before the titanous acid was added, and it was found that under these conditions a considerable loss of hydrogen peroxide occurred, and this in spite of the fact that the catalytic power of the milk had been destroyed by boiling for a quarter of an hour. For instance, in No. 4, the determination of the hydrogen peroxide immediately after its addition showed 94.4 per cent. of the added amount; whereas, after standing overnight, it only showed about 21 per cent. The question naturally suggested itself, whether this disappearance of the hydrogen peroxide from boiled milk might not be due to bacterial action. In order to decide this point, 25 c.c. of milk were treated with 0.01388 gram of hydrogen peroxide, and toluol was added to prevent the growth of bacteria. Another exactly similar mixture was prepared except that no toluol was added. After standing for twenty-four hours, the determination showed in the first mixture about 58 per cent. of the hydrogen peroxide added, in the second about 61 per cent. Therefore it seems that this loss of hydrogen peroxide is not dependent on bacterial growth. In these experiments, the amount of hydrogen peroxide in 5.0 c.c. of milk was too great to permit of a good colorimetric comparison. Therefore 5.0 c.c. of the milk were diluted to 50.0 c.c. with water, and 5.0 c.c. of this diluted milk were used. Harriette Chick¹ noted a certain initial loss of hydrogen peroxide in her experiments, but claims that after this initial loss the hydrogen peroxide content remains constant for a relatively long time. In previously sterilized milk this initial loss was insignificant. Some experiments were made in order to see whether the percentage

¹ Chick, *Centralblatt für Bakteriologie*, vii, 2, p. 705, 1901.

of hydrogen peroxide in milk remains constant after this initial loss. The milk was boiled to prevent catalysis. A mixture containing 0.027 per cent. of hydrogen peroxide still gave the titanous acid reaction after four days, but failed to do so after seven days. A mixture containing 0.054 per cent. hydrogen peroxide reacted faintly with titanous acid after three weeks; but after standing two days longer the reaction was negative. Another specimen of milk containing 0.08 per cent. of hydrogen peroxide was found to be free from it after five weeks. Two specimens of milk containing 0.11 and 0.14 per cent. of hydrogen peroxide still gave the titanous acid reaction after standing for about two months. The apparatus used in our experiments was sterilized; but after the addition of the hydrogen peroxide no effort was made to keep the specimens sterile. It was observed that the milk did not coagulate either spontaneously or on boiling as long as any hydrogen peroxide was present. But after its disappearance, coagulation occurred. This fact holds also for unboiled milk, and must be due to bacterial contamination. Therefore our experiments only show that the amount of hydrogen peroxide added was not sufficient to prevent contamination. This is further illustrated by an experiment in which milk containing 0.03 per cent. of hydrogen peroxide was found to be free from it after thirteen days, while another specimen, to which toluol was added, still gave a strong titanous acid reaction after one month. Two specimens of milk containing 0.012 and 0.015 per cent. lost their hydrogen peroxide in three days, although toluol had been added.

One fact remains. Boiled milk uses up a certain amount of hydrogen peroxide, independently of catalysis and bacterial action. The idea suggests itself, that this may be due to the production of substances on boiling which are capable of reducing hydrogen peroxide. It is known that hydrogen sulphide is liberated when milk is boiled, as was shown by Schreiner¹ and later by Rettger.² Fairley³ demonstrated that hydrogen

¹ Schreiner, Maly, *Jahresbericht über die Fortschritte der Tierchemie*, 1878, p. 146.

² Rettger, *American Journal of Physiology*, vi, p. 450, 1902.

³ Fairley, see Watts's *Dictionary of Chemistry*, Muir and Morley, ii, 1899, under Hydrogen Dioxide.

sulphide slowly reduces hydrogen peroxide with the formation of water and sulphur. It was not determined whether fresh milk is capable of reducing hydrogen peroxide independently of catalysis and bacterial action.

Hecht¹ studied the power of fresh milk (especially human milk) to reduce certain substances in the absence of air. Under these conditions milk is able to reduce methylene blue to its leuco-base.

The opinion of authors who have investigated the value of hydrogen peroxide as a preservative of milk is divided. Schrodtt² experimented with a commercial preparation called Busse's fluid, which contains, besides hydrogen peroxide, 2 per cent. of borax, but his results were not particularly good. Heidenhain³ recommends an addition of 10 per cent. (volume ?) hydrogen peroxide to raw milk, and he states that such milk remains sweet for from three to eight days, and that it can be given to children with impunity. Budde^{4 5} worked out a process in which the hydrogen peroxide is added to milk previously heated to 50° C. The milk is then kept at this temperature for several hours. The milk is heated in order to increase the bactericidal power of the hydrogen peroxide. If there should remain some hydrogen peroxide, it can be removed by means of some catalytic agent. He claims to have obtained very good results with an addition of about 0.035 per cent. of hydrogen peroxide. These statements have been criticised by Barthel⁴ and particularly by Gordan.⁵ Gordan required in his experiments three times the amounts of hydrogen peroxide employed by Budde, and he is of the opinion that the concentration of the hydrogen peroxide is the main factor and that the heating plays a secondary rôle. The concentration of hydrogen peroxide employed by Budde was not able to destroy the peptonizing bacillus of Flügge nor the typhoid bacillus. For the latter a concentration of 0.07 per cent. of hydrogen peroxide proved detrimental.

According to Rosam,⁶ hydrogen peroxide can only effect a

¹ Hecht, *Archiv für Kinderheilkunde*, xxxviii, p. 374, 1904.

² Schrodtt, *Chemisches Centralblatt*, 1884, p. 67.

³ Heidenhain, *Centralblatt für Bakteriologie*, viii, pp. 488 and 695, 1890.

⁴ Barthel, *Milchzeitung*, xxxii, p. 690.

⁵ Gordan, *Centralblatt f. Bakteriologie*, xiii, 2, p. 716, 1904.

⁶ Rosam, *ibid.*, viii, 2, pp. 739 and 769, 1902.

complete sterilization of milk when the milk has been previously heated at 60° – 75° for thirty to forty-five minutes. De Waele, Sugg, and Vandevælde¹ add 0.3–0.4 per cent. of hydrogen peroxide to raw milk and let it stand for several days, when the still undecomposed hydrogen peroxide is catalyzed with sterile laked blood. In this way they claim to have obtained an absolutely sterile milk especially adapted for the use of the infant. Chick states that an addition of 0.2 per cent. of hydrogen peroxide to raw milk insures its permanent sterilization. Our experiments do not seem to bear out this statement, for fresh milk containing 0.28 per cent. of hydrogen peroxide was found coagulated after about two weeks. Rosam speaks of cases where even an addition of 2 per cent. of hydrogen peroxide was not sufficient to sterilize milk. An addition of 0.2 per cent. was not sufficient to keep milk in summer time for a few days. The difference in the results may to a certain extent be due to the variation of the catalytic power of different specimens of milk. The observation of Chick that skimmed milk requires a smaller amount of hydrogen peroxide for sterilization than whole milk, may be partially due to the fact, as shown by Friedjung and Hecht,² that the catalytic power of cream exceeds that of whole milk. Of greater importance seem the following observations. Chick noted in one case that an increase in the number of bacteria took place in spite of the presence of hydrogen peroxide. In our experiments, the growth of a fungus was seen in specimens of milk which still gave a strong reaction for hydrogen peroxide, and this is not an isolated observation. The results of Gordan are very suggestive. He heated milk to 50° for thirty minutes and, after cooling, 0.2 per cent. of hydrogen peroxide was added. In cultures made soon after the addition of hydrogen peroxide, streptococci were found, but no acidifying bacteria. After standing forty-eight hours in the refrigerator, acidifying bacteria were found, and after four days the milk was coagulated. The control was coagulated after standing forty-eight hours in the refrigerator. It seems, therefore, that hydrogen peroxide may inhibit the growth of certain bacteria for some time without destroying them.

¹ De Waele, Sugg, and Vandevælde, *ibid.*, xiii, 2, p. 30, 1904.

² Friedjung und Hecht, *Archiv für Kinderheilkunde*, xxxvii, p. 176, 1903.

The objections which have been raised against the use of hydrogen peroxide as a preservative of milk are the following:

If the usual commercial preparations of hydrogen peroxide of about 3.0 per cent. concentration are employed, the milk is diluted too much, while the use of the more concentrated solutions is too expensive. The peculiar taste which the addition of hydrogen peroxide gives to the milk seems to be objectionable to many persons. On the other hand, Rosam observed that persons who did not like boiled milk took readily his pasteurized milk to which hydrogen peroxide was added. The commercial preparations may be contaminated with injurious substances. Thus, in an analysis cited by Rosam, 0.18 per cent. of barium chloride and 0.0045 per cent. of arsenic were found. The methods requiring heating of the milk are too complicated to be recommended.

The apparent harmlessness of the hydrogen peroxide speaks in its favor. Jablin-Gonnet¹ fed milk containing hydrogen peroxide to young animals and took it himself for two months without any ill effect. Rosam took within a period of about three months an amount of hydrogen peroxide corresponding to 1800 c.c. of a 3 per cent. solution in milk, and did not experience the least injurious effect. The possible action of the hydrogen peroxide on toxins present in milk would be another point in favor of its employment as a preservative. Nadina Sieber² claims that in her experiments, diphtheria and tetanus-toxins, as well as abrin, were neutralized by means of calcium peroxide and also hydrogen peroxide; and Hirschfelder³ succeeded in destroying the toxicity of tuberculin by heating it with hydrogen peroxide, although in this case larger amounts and prolonged heating at high temperatures were necessary. Vandevelde⁴ claims to have shown that hydrogen peroxide favors the action of rennin, pepsin, trypsin, and of the proteolytic enzyme of the milk.

It is undecided whether raw milk can be sterilized by means

¹ Jablin-Gonnet, Maly, *Jahresbericht über die Fortschritte der Tierchemie*, 1901, p. 313.

² Sieber, *Zeitschrift für physiologische Chemie*, xxxii, p. 573, 1901.

³ Hirschfelder, *Journal of the American Medical Association*, 1897.

⁴ Vandevelde, *Beiträge zur chemischen Physiologie und Pathologie*, v, p. 558, 1904.

of hydrogen peroxide in every instance. Furthermore, the quantity of this substance required to act as a preservative for a given length of time is uncertain and seems to vary with different specimens of milk, and under different conditions. But from the experiments it appears that relatively small amounts of hydrogen peroxide, for instance, 0.2-0.5 per cent., frequently suffice to keep milk for a few days at least. If we consider how many infants are denied the benefit of good milk and how many succumb in consequence of this deprivation, particularly in summer time, the use of an apparently harmless preservative at least deserves a trial.

ADDENDA.

The quantitative method above described is not applicable to human milk. Here it is necessary to boil the milk containing the hydrogen peroxide with the acid, in order to effect the coagulation of proteids and to obtain a clear filtrate. This process involves a great loss of hydrogen peroxide. Fresh human milk containing 0.5 per cent. of hydrogen peroxide was found after thirty minutes to contain 0.28 per cent., calculated from the amount of oxygen liberated by the catalyzing action of the milk; whereas the determination of the hydrogen peroxide by means of the titanous acid method showed a content of only 0.05 per cent.

When, in our experiments, the milk (human or cow's) was filtered in order to obtain the filtrate to be used for the colorimetric comparison, it was found that the filter paper was more or less stained blue. This phenomenon occurred whether the milk contained hydrogen peroxide or not, and was independent of the presence of titanous acid. The milk did not contain any formaldehyde. We are unable to account for this interesting color reaction.

When titanium hydrate dissolved in concentrated sulphuric acid was added to milk containing hydrogen peroxide, it was observed that less charring occurred than in the absence of hydrogen peroxide. Some experiments were made to determine the cause of this. The following tables represent the main results of these experiments:

Milk. c.c.	Water. c.c.	Conc. H_2SO_4 . c.c.	Oxidizing substance added.	Remarks.
2.0	1.0	3.0	0.0 c.c. H_2O_2 (10.5 %)	Charred.
2.0	0.0	3.0	1.0 " " "	Light brownish yellow.
2.0	1.5	3.0	0.0 " " "	Charred.
2.0	0.0	3.0	1.5 " " "	Light yellow.
2.0	0.0	3.0	1.0 " KNO_3 (sat'd solution)	Light yellow.

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Milk Sugar solution. (5%) c.c.	Water. c.c.	Conc. H_2SO_4 . c.c.	Oxidizing substance added.	Remarks.
2.0	1.0	3.0		Charred.
2.0	0.0	3.0	1.0 c.c. H_2O_2 (10.5%)	Clear.
2.0	0.0	3.0	1.0 c.c. KNO_3 (sat'd solution)	Clear.

The results of these experiments may be easily explained on the assumption that the addition of the oxidizing substances in general prevents the accumulation of carbon by the action of the sulphuric acid. Whether this is due to the oxidation of the finely divided carbon as it is formed, or whether the whole reaction takes a different course in the presence of the oxidizing agents, was not determined.

SUMMARY.

The titanic acid method of Richardson can be used to determine quantitatively the amount of hydrogen peroxide in milk with approximate accuracy.

Boiled milk uses up a certain amount of hydrogen peroxide. This disappearance of hydrogen peroxide is not brought about by bacterial action, and may be due to the production of reducing substances on boiling, as referred to in the above. It was not decided whether this disappearance also occurs in fresh milk.

While there remains some doubt as to whether hydrogen peroxide can bring about a complete sterilization of raw milk in every case, and while the amount of this substance needed for the preservation of milk during a given period of time is uncertain, its harmlessness seems to justify its trial as a milk preservative.

NOTE.—This paper was sent to the editor of another journal on May 30th. On the 8th of September I was finally informed that the manuscript was lost while on its way to an associate editor. Not having a copy of the paper, it was necessary to rewrite it. This caused considerable delay. In the meantime, an article by Baumann¹ appeared, in which it is stated that hydrogen peroxide even in concentrations of 0.035 per cent. exercises a strong bactericidal power against typhoid, cholera, dysentery, and tubercle bacilli in milk.

¹ Baumann, *Münchener medizinische Wochenschrift*, 1905, No. 23, p. 1083.

SOME CONSIDERATIONS ON PROTEID DIET: WITH ESPECIAL REFERENCE TO ITS CONTENT IN AMIDE-NITROGEN, MELANOIDIN-NITROGEN, DIAMINO-NITROGEN, AND MONAMINO-NITROGEN.

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It is matter of common experience that a given individual may exhibit a variable tolerance for different kinds of proteid food; one may "agree" with him, while another "disagrees." The importance of a search for chemical differences in the food-stuffs, which may be responsible for such differences, is clear. Thus far but little has been done in this direction except (1) the simple determinations of total nitrogen and of elementary percentage composition¹; (2) the interesting studies of Hall and of Burian and Hall² upon the purin-content of the various

¹ Some elementary meat-analyses of various investigators:

	C	H	N	O & S	Ash
Ox flesh:					
(Stohmann and Langbein)	49.25	6.91	15.49	23.03	5.32
(Rubner).....	50.46	7.6	15.4	20.97	5.5
(Argutinsky).....	49.59	6.91	15.31	22.95	5.24
Neck (young ox) (Koehler)..	52.84	7.23	16.41	22.98	0.54
Sirloin (young ox) (Koehler).	52.93	7.12	16.51	22.98	0.46
Sirloin (cow) (Koehler).....	52.22	7.19	16.59	23.42	0.58
Pork (Koehler).....	52.94	7.16	16.51	22.84	0.55
Mutton (Koehler).....	52.69	7.33	16.23	23.09	0.66
Chicken (Koehler).....	52.57	7.10	16.61	23.21	0.51

² "Die Bestimmung der Purinstoffe in tierischen Organen mittels der Methode der korrigierten Werthes," *Zeitschr. f. physiol. Chem.*, xxxviii, p. 336, 1903.

meats,¹ and (3) the studies of v. Fürth² and others upon the complex proteid bodies of muscles.

The object of the research here to be recorded has been to determine comparatively the distribution of the various forms of nitrogen compounds in a series of meats which are ordinarily used as foods. It is well known that proteid substances break down on hydrolysis into a whole series of decomposition products, chief among which are (1) a series of diamino acids, such as arginin, lysin, and histidin; (2) a series of monamino acids, monobasic or dibasic, such as glycocoll, alanin, amino-valerianic acid, leucin, tyrosin, aspartic acid, glutaminic acid, phenylalanin, tryptophan, prolin, oxyprolin, and serin; and (3) ammonia.³ These cleavage products of proteids are believed to be united with one another in the form of long chains

¹ The results of investigations by Schur and Hall's method yield the following figures:

Substance:	Total Purin-Nitrogen in Moist Organ.
Horse flesh.....	0.055 per cent.
Beef.....	0.062 " "
Veal.....	0.071 " "
Thymus (calf).....	0.455 " "
Pancreas (pig).....	0.123 " "
Pancreas (beef).....	0.183 " "

The fact is emphasized that different parts of the body give a different purin-nitrogen content. The neck, cited as an example, is poor in purin. According to the investigations of Hall:

	Purin-Nitrogen.
Neck piece of calf gave.....	0.030 per cent.
Loin of calf gave.....	0.071 " "
Neck piece of pork gave.....	0.023 " "
Loin of pork gave.....	0.048 " "

² "Zur Gewebschemie des Muskels," *Ergebnisse der Physiologie*, I, i, p. 110, 1902.

³ Monamino acids derivable from proteids.

Glycocoll (Amino-acetic acid), $\text{CH}_2(\text{NH}_2).\text{COOH}$.

Alanin (α -amino-propionic acid), $\text{CH}_3\text{CH}(\text{NH}_2).\text{COOH}$.

Leucin (Amino-caproic acid or α -amino-isobutyl-acetic acid), $(\text{CH}_3)_2\text{-CH}.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$.

Phenylalanin (Phenyl-amino-propionic acid), $\text{C}_6\text{H}_5.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$.

Prolin (α -pyrrolidin-carboxylic acid), $\text{C}_5\text{H}_9\text{NO}_2$.

Glutaminic acid (α -amino-n-glutaric acid), $\text{CH}_2\begin{matrix} \diagup \text{CH}(\text{NH}_2).\text{COOH} \\ \diagdown \text{CH}_2.\text{COOH} \end{matrix}$.

in the proteid molecule. Individual proteids are markedly different in their ultimate constitution. The proteids of muscle differ from those of milk; vegetable proteids differ from animal proteids. The variations are quantitative and qualitative. Thus on the one hand, the content in arginin of one proteid may be as much as 83 per cent., of another only 40 per cent., of a third, only 20 per cent., and of a fourth less than 2 per cent. (Kossel); on the other hand, the variety of ultimate constituent amino-acids may vary greatly in the molecules of different kinds of proteid, the relatively simple protamins containing, perhaps, only four or five varieties, while it is known that serum albumin, for example, has as many as seventeen or eighteen different varieties of primary constituents.

A complete knowledge of the chemistry of meats will long be wanting to us, for it will necessitate, among other things, (1) the determination of the different varieties of proteids in each meat; (2) the determination of the fragments into which the proteids of meats break down on digestion and on artificial hydrolysis, and (3) the exact way in which the ultimate constituent fragments are arranged in the molecular chains which the complex molecules represent. Inasmuch as cleavage appears to go far in ordinary digestion, absorption of cleavage products rather than of complex proteid bodies being probable,

Aspartic acid (amino-succinic acid), $\text{COOH}.\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$.

Serin (α -amino- β -oxypropionic acid), $\text{CH}_2(\text{OH}).\text{CH}(\text{NH}_2).\text{COOH}$.

Oxy- α -pyrrolidin-carboxylic acid, $\text{C}_5\text{H}_7\text{NO}_3$.

Tyrosin (p-oxyphenyl- α -aminopropionic acid), $\text{C}_6\text{H}_4(\text{OH}).\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$.

Tryptophan (Skatol- α -amino-acetic acid), $\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_2$.

Amino-valerianic acid, $\text{C}_5\text{H}_{11}\text{NO}_2$.

Cystin (α -diamino- β -dithiodilactylic acid), $\text{COOH}.\text{CH}(\text{NH}_2).\text{CH}_2.-\text{S}-\text{CH}_2.\text{CH}(\text{NH}_2).\text{COOH}$.

Diamino acids derivable from proteids.

Arginin (Guanidin- α -amino-n-valerianic acid), $\text{COOH}.\text{CH}(\text{NH}_2).-(\text{CH}_2)_3.\text{NH}$
 $\begin{array}{c} \diagup \text{CNH} \\ \diagdown \text{NH}_2 \end{array}$

Lysin (α - ϵ -amino-caproic acid), $\text{NH}_2.\text{CH}_2.(\text{CH}_2)_4.\text{CH}(\text{NH}_2).\text{COOH}$.

Histidin, $\text{C}_6\text{H}_7\text{N}_3\text{O}_2$.

the second and third determinations referred to would appear *a priori* to be more immediately useful than the first, though a complete knowledge of (2) and (3) would, in all likelihood, give us a knowledge of (1).

Thanks to a method devised by Hausmann, modified and improved by Osborne and Harris and by Gumbel, we are able to make a preliminary survey of the content of a given proteid (or of a mixture of proteids such as articles of proteid diet represent) in the different kinds of nitrogen-bodies of which they are made up. It is possible to determine relatively easily and approximately accurately the proportions of the total nitrogen of a proteid (or of a meat) which are referable to (1) amide-nitrogen, (2) melanoidin-nitrogen, (3) diamino-nitrogen, and (4) monamino-nitrogen, respectively. The results permit one to compare the holdings in various forms of nitrogen compounds of the different meats with one another, and with those of certain pure proteid bodies which have been studied. It is this method which we have used. The task which we have set ourselves and its results are embodied in the following report.

We have undertaken the determination of the four forms of nitrogen above referred to in the following foods: Pork chops, veal cutlets, sirloin, tenderloin, and neck from the same animal (ox); liver (calf); thymus (calf); heart (calf); chicken (white meat from breast); fish (lake trout).

PREPARATION OF THE MATERIAL—After the removal of excess of fat, the meat in each case was finely minced by means of a sausage-grinder. Dehydration was effected by placing the hash in 95 per cent. alcohol (changed several times), and later in absolute alcohol. The absolute alcohol, after two or three changes, was decanted and ether was added to the residue. In order to remove the fat the material was placed in a large Soxhlet apparatus and extracted with ether for forty-eight hours, at the end of which time the ether washings were tested for a residue of fat. If any was found a longer extraction was made, until all traces of fat disappeared. The material was then dried and pulverized.

For each analysis the powder was dried to constant weight in a weighing tube, in a toluol oven, at 107° C.

METHOD OF ANALYSIS.—The method employed was mainly

that of Hausmann,¹ as modified by Gumbel² and Osborne and Harris.³ The analysis comprises the following steps:

1. The cleavage of the proteid molecule by means of boiling concentrated hydrochloric acid.
2. The estimation of the so-called amide-nitrogen by distillation with magnesia.
3. The determination of the nitrogen occurring in the form of melanoidin, or "humin bodies" carried down in the magnesia paste.
4. The precipitation of the filtrate from the magnesia paste with phosphotungstic acid and the estimation of the nitrogen (diamino or basic nitrogen) in the precipitate.
5. The determination of the nitrogen remaining in the filtrate not driven off by the magnesia nor precipitated by phosphotungstic acid (the monamino-nitrogen).

THE HYDROLYSIS.—About 1 gram of the pulverized meat, dried at 107° until a constant weight was obtained, was boiled with 20 c.c. of concentrated hydrochloric acid (sp. gr., 1.20) in a small flask provided with a reflux condenser for a period of eight to ten hours. The solution was then slowly evaporated on the water-bath or in vacuo, in order to remove a large excess of hydrochloric acid. About 5 to 10 c.c. of a residual solution of cleavage products of the proteid was thus obtained.

THE DETERMINATION OF THE AMIDE-NITROGEN.—The solution of cleavage products was transferred to a distillation flask and about 400 c.c. of water added. Cream of magnesia, prepared by boiling a suspension of the oxide of magnesia for several hours in order to remove every trace of ammonia, was added in slight but distinct excess of neutralization.

The distillation was carried on in vacuo and at a temperature of 40° to 45° C. The ammonia driven off was received into a flask containing a known quantity of decinormal solution of sulphuric acid. From 250 to 300 c.c. was distilled over, usually requiring from three to five hours.

The receiving-flask was kept cool by a stream of running water, or ice, in order to insure condensation of the ammonia vapor, which might otherwise have been drawn off by the vacuum-pump, and caused a low figure for the amide-nitrogen.

If a low boiling-point be not used, the percentage obtained for the amide-nitrogen is apt to be too high, owing to the breaking up of some

¹ "Ueber die Vertheilung des Stickstoffs im Eiweissmolekül," *Zeitschr. f. physiol. Chem.*, xxvii, p. 95, 1899.

² "Ueber die Vertheilung des Stickstoffs im Eiweissmolekül," *Beiträge z. chem. Physiol. u. Path.*, v, p. 297, 1904.

³ "Nitrogen in Protein Bodies," *Jour. Amer. Chem. Soc.*, xxv, p. 323, 1903.

of the monamino acids upon boiling with magnesia at a high temperature. Embden has shown that cystin gives off ammonia on long boiling with magnesia. This source of error is avoided by boiling in vacuo at 40° to 42° C. [at which temperature cystin has been found to give off no ammonia (Gümbel)]. No other cleavage product has been shown to liberate ammonia upon boiling with magnesia.

THE ESTIMATION OF THE MELANIN-NITROGEN (MELANOIDIN OF SCHMIEDBERG).—The "humins" occur as black insoluble masses in the splitting of the proteid by hydrochloric acid. In the analysis they were determined (in the manner followed by Gümbel and Osborne and Harris simultaneously) by determining the nitrogen remaining in the magnesia paste after filtration and complete washing of the paste with distilled water.

The paste was collected on a filter under pressure (over nitrogen-free filter paper), and, after several washings with distilled water, the precipitate, together with the filter paper, was transferred to a Kjeldahl flask and digested with sulphuric acid, and the nitrogen determined according to the method of Kjeldahl. In Hausmann's method this fraction of nitrogen appeared in the diamino precipitate, and correspondingly increased the percentage of the diamino-nitrogen.

THE DIAMINO-NITROGEN.—The filtrate, together with the washings from the magnesia paste, was concentrated in vacuo at 40° C. to about 100 c.c. The precipitation of the diamino acids was effected by means of an acidified solution of phosphotungstic acid of a strength suggested by Osborne and Harris, containing 20 grams of phosphotungstic acid and 5 grams of sulphuric acid per 100 c.c. About 30 c.c. of such a solution was necessary for the complete precipitation of the diamino acids in 1 gram of proteid. The precipitate was allowed to settle, and more phosphotungstic acid was added until a white precipitate no longer appeared in the supernatant fluid upon the addition of a few drops of the precipitant.

Upon standing, the precipitate, which at first is amorphous and flocculent, becomes granular.¹

The precipitate was filtered under pressure through nitrogen-free filter paper and washed with several washings, amounting in all to from 150 to 200 c.c. of a weak solution of phosphotungstic acid. This solution was made up of 2.5 grams phosphotungstic acid and 5 grams of sulphuric acid per 100 c.c.

The precipitate, along with the filter paper, was placed in a beaker and dissolved in a strong solution of sodium hydroxide (27 per cent.). In most cases 50 to 100 c.c. of such solution was used. The filter paper was removed by filtration, and the solution of the phosphotungstates was diluted to a definite volume, usually 500 c.c., or in some cases to a liter.

¹ Gümbel states that crystallization of the diamino phosphotungstates begins in from four to six hours. As a matter of routine it is found best to wait from twenty-four to forty-eight hours before filtering. The criterion in each case is the granular appearance of the precipitate.

An aliquot part of this was taken and the nitrogen in it estimated according to Kjeldahl. From this the percentage of the diamino-nitrogen was determined. About 20 c.c. of sulphuric acid alone, or of Kjeldahl acid, was used for digesting a volume of 200 c.c. of the solution. The oxidation was hastened toward the end by the addition of a few crystals of potassium permanganate.

In the concentration of the filtrate from the magnesia paste to 100 c.c. before precipitation with the phosphotungstic acid a slight brown precipitate frequently was present. This has been observed by Gumbel, who states that nitrogen could never be quantitatively demonstrated in it.

The sources of error in the estimation of the diamino fraction of the nitrogen are due on the one hand to the solubility of the phosphotungstates of the diamino acids (giving too low a percentage) and on the other to the tendency of the monamino acids to be precipitated by the phosphotungstic acid (yielding too high a percentage).

The diamino phosphotungstates dissolve most readily in water, less easily in a solution of acidified phosphotungstic acid, and with still more difficulty if the precipitate be allowed to stand for twenty-four hours, until it becomes crystalline. Hausmann brought his solution after distilling off the ammonia to a volume of 70 to 80 c.c. Gumbel has determined that in a volume of 80 c.c. about .0035 gram of arginin (0.001 N) remains unprecipitated. This in a proteid molecule, with an arginin content of 10 to 20 per cent., gives a loss of 1.8 to 3.5 per cent. This loss is increased by washing the precipitate, even when a dilute acidified phosphotungstic acid solution of the strength given above is used. For this reason as little as possible of the wash solution is used. Lysin is precipitated by phosphotungstic acid in a great dilution (above 1:6000). Histidin precipitates readily with phosphotungstic acid, but redissolves even in a slight excess of concentrated acid. In washing the diamino precipitate with dilute phosphotungstic acid, if too concentrated an acid has been used in the precipitation, the histidin is reprecipitated in the filtrate and necessitates a second filtration; otherwise the histidin will be included in the monamino fraction. Histidin is much less soluble in dilute solutions than are the corresponding precipitates of arginin and lysin. Hence the rule laid down by Gumbel is to use an excess of phosphotungstic acid in precipitating, but not in too high a concentration, since otherwise histidin goes into solution. If a dilution of the fluid is made in which for one part of diamino-nitrogen there are 1000 to 1500 c.c. of solution, the conditions are most favorable for complete precipitation of the diamino acids. Under such conditions the error can be reduced to 5 to 10 per cent. of the total diamino-nitrogen.

The second source of error lies in the precipitation of the monamino acids with phosphotungstic acid. In very concentrated solutions, with the addition of strong acids monamino acids are precipitated with phosphotungstic acid. This does not take place with dilute solutions and with the addition of weak acids, such as are used in the experiment.

According to Winterstein, phenylalanin in a solution of 1 gram in 50 c.c. is no longer precipitated by phosphotungstic acid. Gumbel has shown that a 0.05 per cent. solution of hydrochloric acid of pure albumin-cystin is not precipitated by phosphotungstic acid, even upon standing twenty-four hours. Osborne and Harris state that a careful examination of the phosphotungstic acid precipitates fails to reveal the presence of monamino acids, even when methods were used which would readily show the presence of very small amounts of leucin and tyrosin. They nevertheless conclude that monamino acids are to a slight extent carried down with the phosphotungstic acid precipitate.

DETERMINATION OF THE MONAMINO-NITROGEN.—The filtrate from precipitation of the phosphotungstates was made up to a definite volume (500 or 1000 c.c.). An aliquot portion of it (100 c.c. or 200 c.c.) was taken and digested with 30 c.c. of sulphuric acid, and an estimation of the nitrogen in it made according to Kjeldahl. On account of the high salt content of phosphotungstic acid in this solution, the oxidation is extremely difficult and slow. (For this reason Osborne and Harris have suggested its estimation by subtracting the sum of the other forms of nitrogen from the total nitrogen of the proteid as determined by Kjeldahl.) If the excess of water be removed by slow evaporation, much of the bumping and possible loss of material is avoided, after which the oxidation can be hastened by the occasional addition of a few crystals of potassium permanganate. This oxidation is slow, frequently requiring from twenty-four to thirty-six hours. The actual estimation of this fraction is important, since it serves as a control of the accuracy of the work by permitting a comparison of the sum of the percentages of the different fractions with the percentage of total nitrogen of the proteid in question. It will be noticed that the sums do not quite agree, and the defects of the method (or of technique) are thus made visible.

DETERMINATION OF THE TOTAL NITROGEN.—In each case an estimation of the total nitrogen was made according to the method of Kjeldahl.

It will be noted that the figures obtained refer in all cases to the nitrogen derived from the *article of food as a whole (extracted by alcohol and ether)*.

RESULTS.—The results of the various determinations are presented in the following tables.

The agreements and the differences in these tables are sufficiently striking. How they are to be ultimately valued must be left to further investigations to determine. It is sufficient for the present that we are able to conclude that these various forms of nitrogen compounds are as evenly distributed in articles of proteid diet as they are seen to be, and that they also present in instances very distinct differences from one another, as the tables reveal.

TABLE I.
ESTIMATIONS OF THE AMIDE-NITROGEN.

	Weight of dried material in grams.	Weight of nitrogen in the ammonia distilled with magnesia in grams.	Amide-Nitrogen. Per cent.
Veal cutlets.....	1.1651	0.0119	1.02
Pork chops.....	0.6754	0.0071	1.06
Sirloin.....	0.7708	0.0044	1.04
Tenderloin.....	1.1256	0.0127	1.13
Neck.....	0.9843	0.0090	1.08
Heart.....	0.6641	0.0073	1.09
Liver.....	1.1438	0.0129	1.13
Thymus.....	0.8898	0.0107	1.19
Chicken.....	0.8848	0.0102	1.15
Fish.....	0.6612	0.0074	1.12

TABLE II.
ESTIMATIONS OF THE MELANOIDIN-NITROGEN.

	Weight of dried material used in grams.	Weight of nitrogen estimated by Kjeldahl.	Per cent.
Veal cutlets.....	1.3021	0.0033	0.25
Pork chops.....	0.6754	0.0019	0.29
Sirloin.....	0.7708	0.0029	0.37
Tenderloin.....	0.9931	0.0032	0.32
Neck.....	0.6950	0.0021	0.31
Heart.....	0.6641	0.0026	0.40
Liver.....	1.6539	0.0077	0.46
Thymus.....	0.8898	0.0032	0.36
Chicken.....	0.8848	0.0028	0.31
Fish.....	0.6612	0.0016	0.25

TABLE III.
ESTIMATIONS OF THE DIAMINO-NITROGEN.

	Weight of dried material used in grams.	Volume of the solution of the phosphotungstic acid precipitate in c.c.	Volume used for estimation in c.c.	Weight of nitrogen obtained in aliquot part. Grams.	Per cent.
Veal cutlets..	1.3021	1000	200	0.0097	3.74
Pork chops..	0.6754	1000	200	0.0064	4.77
Sirloin.....	0.7708	500	200	0.0013	4.32
Tenderloin..	0.9931	1000	200	0.0087	4.38
Neck.....	0.6950	1000	200	0.0057	4.13
Heart.....	0.6641	1000	200	0.0054	4.12
Liver.....	1.6539	1000	100	0.0084	5.09
Thymus.....	0.8898	1000	200	0.0113	6.39
Chicken.....	0.8848	500	200	0.0089	4.09
Fish.....	0.6612	500	200	0.0108	4.87

TABLE IV.
ESTIMATIONS OF THE MONAMINO-NITROGEN.

	Weight of dried material used in grams.	Volume of the solution of the phosphotungstic acid filtrate in c.c.	Volume used for estimation in c.c.	Weight of nitrogen obtained in aliquot part in grams.	Per cent.
Veal cutlets..	1.1651	1000	100	0.0040	9.61
" " ..	1.3021	500	200	0.0517	9.63
Pork chops..	0.6754	500	200	0.0275	10.17
Sirloin.....	0.7708	500	200	0.0297	9.65
Tenderloin..	0.9931	500	200	0.0402	10.09
Neck.....	0.6950	500	200	0.0299	10.75
Heart.....	0.6641	500	200	0.0273	10.30
Liver.....	1.1438	1000	200	0.0228	10.00
"	1.1438	1000	100	0.0116	10.19
"	1.6539	1000	100	0.0157	9.50
Thymus.....	0.8898	500	200	0.0280	7.87
Chicken.....	0.8848	500	200	0.0372	10.51
Fish.....	10.69

TABLE V.
DISTRIBUTION OF NITROGEN EXPRESSED IN TERMS OF THE WEIGHT.

		Amide-nitrogen, per cent.	Melanoidin-nitrogen, per cent.	Diamino-nitrogen, per cent.	Monamino-nitrogen, per cent.	Sum of components.	Total nitrogen estimated by Kjeldahl.
Ox, same animal.	Veal cutlets.....	1.04	0.25	3.74	9.62	14.65	16.04
	Pork chops.....	1.06	0.29	4.94	10.17	16.46	15.44
	Sirloin.....	1.04	0.37	4.32	9.65	15.38	15.44
	Tenderloin.....	1.13	0.32	4.38	10.09	15.92	15.05
	Neck.....	1.08	0.31	4.13	10.75	16.27	15.49
	Heart (calf).....	1.09	0.40	4.12	10.30	15.91	15.37
	Liver (calf).....	1.13	0.46	5.09	9.89	16.57	15.16
	Thymus (calf).....	1.19	0.36	6.36	7.87	15.78	15.72
	Chicken.....	1.15	0.31	4.09	10.51	16.06	15.85
	Fish.....	1.12	0.25	4.87	10.69	16.93	15.08

TABLE VI.
DISTRIBUTION OF NITROGEN EXPRESSED IN PERCENTAGES OF THE TOTAL NITROGEN.

		Amide-nitrogen.	Melanoidin-nitrogen.	Diamino-nitrogen.	Monamino-nitrogen
Ox, same animal.	Veal cutlets.....	7.10	1.71	25.53	65.65
	Pork chops.....	6.43	1.76	30.01	61.78
	Sirloin.....	6.76	2.40	28.08	62.72
	Tenderloin.....	7.09	2.01	27.51	63.38
	Neck.....	6.65	1.91	25.46	65.98
	Heart (calf).....	6.85	2.51	25.89	64.73
	Liver (calf).....	6.82	2.78	30.72	59.68
	Thymus (calf).....	7.54	2.28	40.31	49.87
	Chicken.....	7.16	1.93	25.46	65.44
	Fish.....	6.62	1.48	28.47	63.14

ACETONURIA FOLLOWING CHLOROFORM AND ETHER ANÆSTHESIA.

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The pronounced degenerative changes which may occur in the liver after chloroform narcosis were recognized as early as 1866 by Nothnagel,¹ and have been noted and carefully studied by a number of observers since his time. More recently the symptoms of these cases of chloroform poisoning have been studied by Guthrie,² Stocker,³ Brewer,⁴ Brackett,⁵ Kelly,⁶ and Bevan and Favill.⁷ These symptoms have been recognized as those of an acid intoxication and are described by Bevan and Favill as follows: "This symptom-complex consists of vomiting, restlessness, delirium, convulsions, coma, Cheyne-Stokes respiration, cyanosis, icterus in varying degree, and usually terminates in death."

Out of twenty-eight cases of death following chloroform anæsthesia and having these characteristic symptoms, which are noted in Bevan and Favill's paper, the urine was tested in four instances for the presence of acetone and diacetic acid and these bodies were found. In another case there was noted an acetone-like odor to the breath, and in another leucin, tyrosin, and bile pigment were found in the urine.

A routine examination of the urine for acetone, in the cases of patients who had been anæsthetized, was made by Greven⁸ in 1895, but the value and significance of these observations seem

¹ Nothnagel, *Berl. klin. Wochenschr.*, iii, p. 31, 1866.

² Guthrie, *Lancet*, i, p. 193, 1894.

³ Stocker, *Centralbl. f. Gynäk.*, No. 45, 1895.

⁴ Brewer, *Ann. of Surg.*, xxxvi, p. 481, 1902.

⁵ Brackett, Stone, and Low, *Boston Med. and Surg. Journ.*, cli, p. 2, 1904.

⁶ Kelly, *Ann. of Surg.*, xli, p. 61, 1905.

⁷ Bevan and Favill, *Journ. of Am. Med. Assoc.*, xlv, pp. 691 and 754, 1905.

⁸ Greven, *Ueber Acetonurie nach der Narkose*. Bonn, 1895.

to have escaped any widespread notice. Greven studied 251 cases of anæsthesia under chloroform, ether, and bromethyl, and found that acetone appeared in the urine in 167 cases. In many of these examinations he tested the urine directly without distilling, which is a less accurate method than Lieben's iodoform test applied to the distillate.

In the report of Brewer's case of chloroform poisoning, he states that he has had routine examinations made in thirty-three cases of anæsthesia from ether and chloroform and that acetone was found in seven of these cases. Since then Dr. Brewer has had further examinations made, the summary of which he has allowed me to report with the present series of cases. He had an examination for acetonuria made in the case of seventy-eight patients in addition to the thirty-three mentioned above. In sixty-four cases, where the examination was made on the day following operations, the acetone reaction was well marked in fifty-nine. In ten cases not examined till the second day after the operation, there were seven positive results. In two cases not examined till the third day after the operation, there were two negative results.

In several cases where acetone was found immediately after operation, it persisted a number of days: in one case for seven days and in two cases for ten days.

In twelve cases of postoperative acetonuria, tests were made for diacetic acid with one positive result.

Dr. Brewer concludes from his cases that in at least ninety per cent. of cases there is a well marked acetonuria during the first few hours after anæsthesia.

As it has been generally considered that, barring some idiosyncrasy or the presence of some organic disease, the giving of chloroform and ether does not produce any really unfavorable effect upon the patient, it has seemed well to make further routine examinations regarding the changes occurring in the urine after the use of anæsthetics. The following report relates to the analysis of the urine in forty-one cases of anæsthesia occurring in the service of Dr. Brewer at the Roosevelt Hospital. These patients were anæsthetized with chloroform or ether or with a combination of the two. The results of the analyses are given in the accompanying tables.

TABLE I.

Operation.	Relation to Time of Operation.	Anesthetic.	Amount of Anesthetic Used.	Duration of Anesthesia.	Sp. Gr.	Reaction.	Volume in c.c.	Acetone.	Organic Acid in Terms of NaOH.	Phenol.	Indicator.
Reversion of Cecum and Ascending Colon	Before. After.	Ether.	500 c.c.	2 hrs. 7 min.	1037	Strongly acid. Alkaline.	430 930	Trace.	12.7 26.	Faint trace. Distinct reaction.	
Double Bassini.	Before. After.	Ether.	250 c.c.	45 min.	1028 1035	Very strongly acid. Acid.	310 400	Faint trace.	10.0 14.4	Distinct reaction.	
Excision of Varicose Veins.	Before. After.	Ether.	435 c.c.	1 hr. 20 min.	1028 1039	Faintly acid. Alkaline.	250 480	Large amount.	5. 10.	Faintest trace. Trace.	0 0
Hemorrhoids.	Before. After.	Chloroform.	31 c.c.	15 min.	1022 1029	Strongly acid. Strongly acid.	830 400	0 0	23.0 7.4	Distinct reaction. Trace.	0
Low Tachyotomy.	Before. After.	Chloroform and Ether.	245 c.c.	30 min.	1022 1040	Very strongly acid. Very strongly acid.	500 150	Large amount.	8.8 5.5	Trace. Distinct trace.	0
Plastic Operation on Mouth.	Before. After.	Chloroform.	8 c.c.	35 min.	1018 1021	Strongly acid. Alkaline.	500 810	Large amount.	20. 17.4	Trace.	0
Hemorrhoids.	Before. After.	Ether.	250 c.c.	20 min.	1038	Alkaline. Strongly acid.	1000 410	Very large amount.	28.2 0.6	Faint trace. Trace.	
Raynaud's Disease. Amputation of Leg.	Before. After.	Chloroform and Ether.	180 c.c.	50 min.	1038	Strongly acid.	700 475	0	57.7 10.3	Marked reaction. Moderate amount.	Distinct reaction.
Varicose Veins.	Before. After.	Ether.	180 c.c.	25 min.	1038	Strongly acid.	500	0	21.0	Present.	0
Tubercular Adenitis.	Before. After.	Chloroform.	60 c.c.	40 min.	1020 1035	Acid.	200 610	Faintest trace.	0.30	Trace.	
Resection of Wrist. Tuberculosis.	Before. After.	Ether.	220 c.c.	45 min.	1038 1032	Strongly acid. Strongly acid.	200 460	Large amount.	13.1 21.4	Large amount. Faint trace.	0
Intermuscular Appendicectomy.	Before. After.	Chloroform.	15 c.c.	12 min.	1019 1030	Alkaline.	220 460	Very faint trace. Considerable amount.	14.7 10.0	Faint trace. Distinct trace.	
Cervical Adenitis.	Before. After.	Ether.	250 c.c.	55 min.	1012 1015	Very strongly acid. Strongly acid.	330	Faint trace.	0	Faint trace. Large amount. Present. Less than before.	0
	Before. After.	Ether.	200 c.c.	45 min.	1013 1013	Alkaline. Very strongly acid. Strongly acid. Very strongly acid.	330	Very large amount. Considerable amount. Large amount.	10.34		

TABL inued.

Operation.	Relation to Time of Operation.	Anesthetic.	Amount of Anesthetic Used.	Duration of Anesthesia.	Sp. Gr.	Reaction.	Volume in c.c.	Acetone.	Organic Acid in Terms of 10^{-10} NaOH.	Phenol.	Indican.	Diacetic Acid
Varicose Veins.	Before. After.	Chloroform and Ether.	2 c.c. 300 c.c.	1 hr. 5 min.	1043 1040	Alkaline. Acid.	320 370	Trace. Large amount.		Marked reaction. Less than before.		0
Varicocele.	Before. After.	Chloroform	45 c.c.	27 min.	1018 1047	Alkaline. Alkaline.	580 610	0 Large amount.	6.6	Very marked. Faintest trace.		
Varicocele.	Before. After.	Chloroform	15 c.c.	20 min.	1030 1032	Very strongly acid. Acid.	630 440	0 Large amount.	12.5	0 Large amount.		
Appendicectomy.	Before. After.	Chloroform and Ether.	1 c.c. 60 c.c.	25 min.	1036	Strongly acid.		Large amount.		Trace.		
Inguinal Hernia.	Before. After.	Ether.	290 c.c.	40 min.	1028 1035	Alkaline. Strongly acid.	420 420	0 Large amount.		Trace. Marked reaction.		
Hydrocele.	Before. After.	Ether.	500 c.c.	40 min.	1025 1035	Alkaline. Strongly acid.	560 420	0 Large amount.		Large amount. Large amount.		
Cellulitis of Hand.	Before. After.	Ether.	125 c.c.	20 min.	1012 1023	Alkaline.	180 430	Trace. Trace.		Distinct reaction. Trace.		
Appendicectomy.	Before. After.	Ether.	290 c.c.	25 min.	1035 1040	Alkaline. Alkaline.	300 470	Considerable amount. Trace.		Marked amount. Trace.		
Hernia.	Before. After.	Ether.	250 c.c.	25 min.	1035 1034	Strongly acid. Alkaline.	820	Considerable amount.		Distinct trace. Trace.		0
Exploratory Laparotomy.	Before. After.	Ether.	290 c.c.	25 min.	1026 1035	Alkaline. Strongly acid.	150	0 Large amount.		Trace. Considerable amount.		0
Femoral Hernia.	Before. After.	Ether.		36 min.	1013 1018	Acid. Acid.	1080 ?	0 Distinct reaction.		Marked reaction. Distinct trace.	0 0	
Fracture of Patella. Nephritis.	Before. After.	Ether.		23 min.	1014 1017	Alkaline. Alkaline.	960 450	0 Slight amount.		Very marked reaction. Very marked reaction.	0 0	
Hernia. Bassini's Operation.	Before. After.	Ether.	249 c.c.	1 hr.	1013 1020	Strongly acid. Strongly acid.	340 320	0 Distinct trace.		Faint trace. Trace.		
Cleft Palate. Adenoids.	Before. After.	Ether.	250 c.c.	1½ hr.	1022 1029	Strongly acid. Strongly acid.	360 230	Marked reaction. Very marked reaction.		Trace. Large amount.		
—	Before. After.	Ether.	250 c.c.	24 min.	1017 1031	Strongly acid.	200 680	0 Large amount.		Faint trace. 0		

Impacted Falces	Before After	Ether	185 c c	30 min	1031 1032	Very strongly acid Very strongly acid	380 475	Trace Distinct trace.	12.1 31.7	Very strong reaction. Less than before.	0
Inguinal Adenitis	Before After	Chloroform	10 c c	1 hr 10 min.	1033	Alkaline Alkaline	850 310	Present.	57.5 7.2	Distinct trace	
Intermuscular Appendicectomy.	Before After.	Chloroform	18 c c	13 min	1015	Strongly acid Very strongly acid	1000 720	Distinct reaction. Large amount	21.2 20.	Trace.	0
Cholelithiasis.	Before After	Ether	135 c c	2 hrs	1021 1020	Strongly acid Very strongly acid	830 200	Trace. 0		Trace. Trace.	
Enucleation of Thyroid	Before After	Ether	220 c c	25 min	1035	Strongly acid Strongly acid	550	Large amount		Large amount Faint trace	
Ventral Hernia	Before After	Ether	220 c c	40 min	1020	Strongly acid Strongly acid	600	Considerable amount. Large amount		Trace 0	0 0
Thyroid Cyst	Before After	Ether	100 c c	30 min	1030 1020	Strongly acid Strongly acid	370 530	Trace Very large amount		Trace Faint trace.	
Appendicitis	Before After	Ether		11 min	1030 1021	Strongly acid Alkaline	600 610	Trace. Marked reaction		Distinct reaction. Trace.	0 —
Appendix Disease	Before After	Ether		11 min	1017 1038	Alkaline Alkaline	720 600	Faint trace Very large amount.		Distinct trace Faint trace.	Faint trace. 0
Appendicectomy Ventral Suspension Resection of R Ovary	Before After	Ether	300 c c	1 hr 15 min	1020 1032	Strongly acid Very strongly acid	715	Trace Very marked.		Large amount. Trace.	0
Pain Kidney.	Before After	Chloroform	45 c c	35 min	1015 1021	Alkaline Alkaline		Considerable amount Considerable amount		Large amount. Distinct trace.	0
Stricture of Rectum Dilatation	Before After	Ether.	180 c c	20 min.	1022 1022	Alkaline Alkaline		Trace Trace		Faint trace. Marked reaction.	0

TABLE II.
(CASES 27-34 OF TABLE I.)

Operation.	Relation to Time of Operation.	Sulphuric Acid Preformed.	Sulphuric Acid Ethereal.	Ratio $\left\{ \begin{array}{l} \text{H}_2\text{SO}_4 \text{ Preformed} \\ \text{H}_2\text{SO}_4 \text{ Ethereal.} \end{array} \right.$	Urea.	Nitrogen.	Nitrogen of Ammonia. Grams.	Nitrogen of Ammonia. Per Cent. of Total N.
Hernia (Bassini)	Before	0.2595	.0395	6.5	3.8	1.77	.1111	6.2
	After	0.4748	.0565	8.4	7.718	3.60	.3046	8.4
Cleft Palate Adenoids	Before	1.065	.0820	13.	15.4	7.19	.5356	7.4
	After	0.7981	.0661	12.	6.882	3.21	.0157	3.9
	Before	0.4436	.0426	10.4	4.764	2.22	.0972	2.1
	After	1.9763	.0917	20.4	23.06	10.8	.4831	4.5
Impacted Fæces	Before	0.9667	.5693	1.7				
	After	1.2533	.5907	2.1				
Inguinal Adenitis	Before	1.690	.1950	8.6				
	After	0.7576	.0813	9.3				
Intermuscular Appendicectomy	Before	2.2545	.1935	11.6				
	After	2.2109	.0101	21.8				
Cholelithiasis	Before	1.9112	.1328	14.4	16.87	7.88	.8318	10.5
	After	0.7441	.0424	17.5	10.85	5.07	.349	6.8
Enucleation of Thyroid	Before							7.
	After				23.9	11.15	.8112	7.28
Ventral Hernia	Before							9.6
	After				16.3	7.61	.4742	6.23
Thyroid Cyst	Before	1.0841	.0469	20.9				
	After	1.4286	.0704	20.3				

In studying these tables it will be noted that in all but two of these cases, acetone¹ was excreted in the urine during the first twenty-four hours after the operation. In eight of these cases only a trace of acetone was found, and in the remaining twenty-

¹ The following method was used in testing for acetone: 5 c.c. of strong H_2SO_4 were added to 100 c.c. of urine which was then distilled. About 60 c.c. of the distillate were collected. A portion of this was warmed in a test-tube and rendered alkaline by the addition of a few drops of a solution of potassium hydroxide. Then Lugol's solution of

nine (or 70 per cent.) there was a marked reaction. In twenty-eight cases ether alone was used. In eight of these there was a trace of acetone after the operation, and in twenty there was a marked or very marked reaction. In nine cases chloroform alone was used. In one of these there was no acetone excreted in the urine; in two cases there was a trace and in six there was a marked reaction. In four cases chloroform and ether were both used, and of these one had no acetone and three had a marked reaction. It would appear from these observations that a marked reaction for acetone is as likely to occur after ether as after chloroform anæsthesia. The average duration of the operations in which chloroform alone was used was thirty-three minutes, that in which ether alone or ether and chloroform were used was forty-six minutes.

The duration of the anæsthesia did not have as much effect upon the amount of acetone excreted as might have been expected. The effect of the length of the operation may be seen in the following summary:

Duration of operation.	Acetone reaction.			
	None.	Trace.	Marked.	Very Marked.
30 min. or less..	1	8	9	3
30 to 60 min. . .	1	1	8	4
Over one hour. .	0	3	0	3

In only two cases was the test for acetone continued after the first twenty-four hours following the operation, and in both of these there was still a marked reaction on the second day, but much less on the third. Greven found in his cases that the acetone usually disappeared from the urine within a few days after the operation.

The urine of each patient was tested for acetone before the operation. In six cases acetone was present. In the rest there was none found or only a trace which was not more than a iodine was added drop by drop until there was a slight excess. The solution of potassium hydroxide was again added, drop by drop, until the brown color disappeared. With this test, if acetone is present, there is a prompt precipitation of iodoform.

physiological amount. The conditions in which acetone was found before operation were appendicitis (two cases), thyroid tumor, adenitis, hydrocele, and abscess of the kidney. In three of these acetone was definitely increased after the operation. In two, appendicitis and enlarged thyroid, the acetone was less after the operation than before.

As it has been claimed that the giving of carbohydrate food greatly lessens the amount of acetone in the urine, in pathological conditions, or causes its disappearance, rather large quantities of sugar were given to six of these patients before the operation to discover whether it would prevent the appearance of acetone after the narcosis. Two patients received 25 grams of cane-sugar, three received 75 grams, and one, 150 grams. The giving of the sugar did not appear to affect the amount of acetone excreted. In all these cases receiving sugar, there was a distinct reaction for acetone after the anæsthesia; in three cases the reaction was marked, and in one very marked.

An examination for diacetic acid¹ was made in fifteen cases in the urine excreted during the first day after the operation, and in all of these the result was negative. In two cases, however, a test was made for diacetic acid in the urine collected on the second and third day after the operation, and in both cases it was found in these later specimens.

The presence in the urine of these patients of certain other organic acids, including aromatic oxy-acids and hippuric acid (see table), was tested by adding sulphuric acid and extracting the urine with ether. After washing this extract to remove all traces of sulphuric acid, the residue was titrated with decinormal sodium hydroxide solution to determine the acidity. Fourteen of the specimens collected before and after the operation were thus examined. The average excretion of organic acid thus determined was, in the specimens before the anæsthesia, equivalent to 25.4 c.c. of a decinormal solution of sodium hydroxide. The average of the specimens collected after the anæsthesia was equivalent to 13.5 c.c. of the same solution.

¹In testing for diacetic acid a solution of ferric chloride was added in a slight excess. If the Bordeaux-red color appeared, another portion of the urine was boiled, after the addition of a few drops of sulphuric acid, and again tested with ferric chloride.

In six cases the nitrogen of ammonia¹ in the urine was estimated before and after the operation. The average nitrogen of ammonia excreted in the specimens collected before the operation was 7.1 per cent. of the total nitrogen, and in the specimens after anæsthesia 6.2 per cent. of the total nitrogen. The variations in the different cases are such that no definite conclusions can be drawn from so few cases. That there is no tendency in the first twenty-four hours for the nitrogen of ammonia to increase is noteworthy, as it corresponds to the evidence that there is no increase in organic acids.

A number of the specimens of urine were tested for indications of the degree of bacterial activity in the bowel before and after the operation. It seemed especially desirable to do this, for the reason that a paralytic condition of the intestines has often been suspected or observed after narcosis, and it would be reasonable to suppose that this disturbance of function and the diminished secretion of digestive juices which is supposed to occur might lead to an increased production and absorption of putrefactive products. There has been a disposition to attribute to intestinal putrefactive products symptoms which are now being referred with a high degree of probability to the influence of the anæsthetics upon the cells of parenchymatous organs. In thirty-five cases a test was made for phenol.² Of these, twenty excreted less phenol after the anæsthetic than before, five had the same amount before and after the operation, and ten showed more after the anæsthesia. In only eight cases was a test made for indican,³ and in six of these there was no indican in the urine either before or after the operation. Two that had a marked reaction before showed less after the operation.

In eight cases an examination was made to determine the ratio of the preformed to the ethereal sulphates,⁴ before and

¹ The nitrogen of ammonia was estimated by Schlösing's method.

² The distillate from the acidified urine was tested with Millon's reagent for phenol.

³ To test for indican, equal parts of urine and a solution of ferric chloride in fuming hydrochloric acid were mixed. The mixture was then agitated with a few drops of chloroform which takes up the blue pigment.

⁴ The preformed and ethereal sulphates were estimated by Salkowski's gravimetric method.

after the operation. This ratio was eleven to one before, and fourteen to one after, the operation. Neither these ratios nor the totals point to an increase in ethereal sulphates immediately after narcosis.

Although we thus note that in these cases there was no increase in the intestinal putrefaction following the narcosis, the study of the putrefactive products in persons subjected to anæsthesia may nevertheless prove to be a matter of very great importance in connection with increased susceptibility to the toxic action of narcotics. For it is conceivable that chronic states of putrefaction, which have led in the course of time to impaired liver function, may have a real part in explaining the susceptibility of exceptional patients to chloroform and ether.

The characteristics of the urine after anæsthesia, as shown in this limited number of cases, are a higher specific gravity, a more strongly acid reaction, and an excretion of acetone, which in seventy per cent. of the cases gave a marked reaction. The organic acid was usually less than before the operation and the excretion of ammonia less, while the products of intestinal putrefaction were not materially altered, being slightly diminished.

The point of especial interest in this series of observations is the confirmation of Greven's discovery, that the routine examination of the urine following anæsthesia shows that in almost every case there is enough disturbance in metabolism, probably in the liver cells, to cause the appearance of a distinct or marked reaction for acetone in the urine. It is also noteworthy that in this limited number of observations this mild degree of metabolic disturbance is as likely to follow ether as chloroform anæsthesia, and does not bear any definite relation to the amount of the anæsthetic used, but may give a marked reaction after a short operation where a small amount of the ether or chloroform is given.

The observations recorded here relate to cases in which narcosis was followed by no untoward results referable to the anæsthetic, and may therefore be regarded as typical of normal anæsthesia by chloroform and by ether.

I wish to acknowledge my obligation to Dr. Herter at whose suggestion this study was made, to Dr. Brewer for kindly placing his patients under observation, and to Drs. Coerr and Hervey for making careful records of the clinical features in these cases.



ON A RELATION BETWEEN SKATOL AND THE DIMETHYL-AMIDOBENZALDEHYDE (PARA) REACTION OF THE URINE.

By C. A. HERTER.

(Received for publication, November 16, 1905.)

In 1901 Ehrlich¹ showed that most urines have a constitution which is capable of entering into color reactions with p-dimethylamidobenzaldehyde (Ehrlich's aldehyde). The reagent is employed in acid solution, generally with the aid of heat, and the cherry-red produced fades to orange-yellow on standing (rapidly in the presence of excess of acid), and is partly soluble in chloroform and completely so in epichlorhydrin. The appearance of the color is prevented by treating the urine with an aliphatic aldehyde like formalin, probably because the reacting substance in the urine is firmly united to the aldehyde and thus prevented from combining with the color-producing, aromatic aldehyde. It was noticed by Ehrlich that the intensity of the reaction is apt to be great in pathological urines, including those from patients with phthisis, typhoid fever, and chronic enteritis, and Clemens observed that persons with digestive disturbances are among those whose urines are likely to give a strong reaction. It has not been possible, however, for those who have heretofore worked on the subject, either to attach to the Ehrlich aldehyde reaction any definite clinical significance or to discover the chemical nature of the substance in the urine on which it depends. Still it appears that observers are agreed that in its intense form the reaction is pathological. Hence any light on the nature of the reaction is of medical as well as of biological interest.

While engaged in testing the urines of various persons with the Ehrlich aldehyde, it appeared to me that the urines of persons with urinary evidences (excess of indican and phenol) of exaggerated intestinal putrefaction are especially liable to exhibit

¹ *Medicinische Woche.*, 1901, No. 15.

the deepest cherry-color tints on being treated with the reagent, and that the urines of normal children (whose intestinal putrefactive processes are apt to be mild) often gave negative or almost negative results. This observation suggested the desirability of determining whether the intensity of the reaction is influenced by the presence of derivatives of putrefactive substances in the urine. Before making experiments in this direction, it was decided to determine whether the introduction of large amounts of free hæmoglobin simultaneously into the blood and into the intestine is followed by the intensification of the Ehrlich aldehyde reaction of the urine, due to the formation of a biliary chromogen (urobilinogen) and perhaps hæmopyrrol.¹ A dog of medium size was bled 150 c.c. and the defibrinated blood laked with distilled water. One-half the laked blood was infused intravenously and the other half was put in the stomach of the dog. The urine remained negative so far as the Ehrlich aldehyde reaction was concerned.

Experiments were next made with indol and skatol. Indol is well known to give a pink condensation product with Ehrlich's aldehyde. Skatol gives a violet or blue condensation product with the substance. The administration of 0.1 gram of indol to a dog was not followed by any alteration in the Ehrlich aldehyde reaction of the urine.

Experiments with skatol (Kahlbaum's) showed that the administration of the substance by the gastro-enteric path or subcutaneously regularly exerts a definite influence on the Ehrlich aldehyde reaction of the urine, as the following notes indicate.

Experiment 1.—Normal dog, weight about 18 lbs. Urine negative to Ehrlich's aldehyde. No reaction for skatol with concentrated hydrochloric acid. Bladder emptied. Gave 0.1 gm. skatol by stomach. Urine collected 1 hour later by catheter gives fairly strong cherry-red color with Ehrlich's aldehyde. The color goes over readily into chloroform. Urine collected by catheter three hours after skatol gives a moderate red with Ehrlich's aldehyde, which shakes out in chloroform. With concentrated

¹ This experiment was suggested by the views of Neubaur on the relation of hæmopyrrol (methylpropylpyrrol) to Ehrlich's reaction. See O. Neubaur, *Sitzungsbericht der Gesellschaft f. Morphologie u. Physiologie*, 1903, 2, p. 32.

hydrochloric acid the urine gives a strong skatol red reaction, but requires heat to bring it out. Urine collected by catheter five hours after skatol gives with Ehrlich's aldehyde a pink red, quickly becoming dirty brown, which shakes out moderately strong yellow red in chloroform.

Experiment 2.—Rhesus monkey (No. 1), weight about 5 lbs. Normal urine collected from cage and filtered. Sp. gr., 1.017. With Ehrlich's aldehyde gives slight reaction with light pink color. No skatol red reaction with cold concentrated hydrochloric acid. Received 0.1 gm. skatol subcutaneously. Urine of following seventeen hours collected from cage and filtered. Sp. gr., 1.025. Gives intense cherry-red reaction with Ehrlich's aldehyde. After dilution to sp. gr. 1.014 still gives a marked cherry-red reaction. On standing, this urine gives a strong skatol red reaction with cold concentrated hydrochloric acid.

Subsequent urines showed a falling off in the intensity of the aldehyde reaction. A urine collected from cage about forty-eight hours after the skatol injection, diluted to sp. gr. 1.016, gave a moderately strong Ehrlich reaction. Concentrated hot hydrochloric acid gave a slight skatol red reaction. A second injection of 0.1 gm. skatol was followed after eight hours by collection of a urine which, after dilution to sp. gr. 1.015, gave an intense cherry-red with Ehrlich's aldehyde. On cooling suddenly there occurred a brownish precipitate. Chloroform takes out a red coloring matter, leaving behind a purplish material. Formic aldehyde quite prevents the reaction. Concentrated hydrochloric acid gives a strong skatol red.

Urine collected twenty-four hours after the second injection still gives a strong aldehyde reaction. Sp. gr., 1.013.

Urine collected forty-eight hours after the second injection gives a slight Ehrlich reaction. Sp. gr., 1.015.

Urine collected four hours after a third injection of 0.1 gm. skatol gives an intense cherry-red Ehrlich's reaction. Sp. gr. not taken.

Urine collected eight hours after the third injection gives an extremely intense ruby-red color with Ehrlich's reagent.

Experiment 3.—Rhesus monkey (No. 2). Urine collected from cage, sp. gr., 1.020, diluted to 1.013, gives slight reaction with Ehrlich's aldehyde. Thirty hours after subcutaneous injection of 0.1 gm. skatol, urine diluted to 1.013 sp. gr. gives strong to intense Ehrlich's aldehyde reaction. Urine collected forty-eight hours after injection of skatol, diluted to sp. gr. 1.018, gives intense Ehrlich reaction. Urine collected seventy-two hours after injection (sp. gr., 1.011) gives moderate Ehrlich reaction. Urine collected ninety-six hours after injection (sp. gr., 1.017) gives moderately strong Ehrlich reaction and slight skatol red with concentrated hydrochloric acid (with slight heat).

Urine collected five hours after second injection of 0.1 gm. skatol subcutaneously (sp. gr., 1.012) gives intense cherry-red reaction with Ehrlich's aldehyde. The color goes over completely into epichlorhydrin. The reaction is checked by formic aldehyde. The urine gives an intense red with concentrated hydrochloric acid (with slight heat).

Experiment 4.—On Nov. 6, at 10 A.M., a healthy man passed urine (sp. gr., 1.020) giving a marked reaction with Ehrlich's aldehyde. He then took 0.025 gm. skatol (in solution) by the mouth, and at 1 P.M. this dose was repeated. At 1 P.M. urine was passed and diluted to sp. gr. 1.020. Ehrlich's reaction was of about the same intensity as in the preceding urine. The skatol red reaction was stronger. Urine passed at 3.15 P.M. (sp. gr., 1.026) was diluted to sp. gr. 1.023 and gave an intense Ehrlich reaction (distinctly more intense than in previous urines). Urines subsequently passed gave a strong or moderate Ehrlich reaction. During the morning of Nov. 8, the subject took 0.050 gm. skatol. Urines passed about two hours and about four hours after the last dose of skatol gave intense Ehrlich reactions. Urines passed subsequently gave weaker reactions, but on the following day a urine was obtained which gave an intense reaction.

Experiment 5.—Urine from a normal man collected at 8 A.M., Nov. 19, gave a slight reaction with Ehrlich's aldehyde—almost negative. Urine collected at 10.45 A.M. also gave a slight reaction. At 10.40 A.M. the subject took 0.05 gm. skatol in gelatin capsule with water. No symptoms were noted.

Urine secreted from 12 M.—4 P.M. gives marked reaction.

"	"	"	4 P.M.—6 P.M.	"	"	"
"	"	"	6 P.M.—8 P.M.	"	"	"

All the urines used in the above tests were diluted with water to sp. gr. 1.010. The increase in the intensity of the reaction after taking skatol was unquestionable.

The experiments here described show clearly that the administration of skatol was followed regularly by an intensification of the Ehrlich aldehyde reaction of the urine, and this result was especially striking in the case of Experiment 2 and Experiment 3. The intensified reaction did not differ from the spontaneous reaction in respect to the partial solubility of the cherry-red color in chloroform and the complete solubility in epichlorhydrin. It also resembled the spontaneous reaction in being abolished by the action of formic aldehyde. Whether the intensified color following upon the administration of skatol has the same spectroscopic characters as the spontaneous color reaction I am unable to say. There was in all the experiments a rough correspondence between the Ehrlich reaction and the reaction with concentrated hydrochloric acid, known as the skatol red or urorosein reaction, but no close parallelism between the former and the reaction of indican.

The urine of the subject of Experiment 4 habitually gives a

tents in relation to the behavior of the urine with Ehrlich's aldehyde.¹

The tests of the urine were made for the most part with the aid of heat, although pronounced differences were also observed when the reaction was carried out in the cold.

Note during proof-reading.—Since the above was written, I have found that in persons whose urines give a strong Ehrlich reaction the feces contain skatol, but that in persons whose urines are negative to the aldehyde there can be recovered little or no skatol from the feces. No exception to this correspondence has yet been observed, and I am disposed to think that the absorption of skatol from the intestine is a common cause of the aldehyde reaction. The behavior of the urines to concentrated hydrochloric acid also supports this view.

I have further observed that the subcutaneous injection of tryptophane in monkeys causes an increase in the Ehrlich aldehyde reaction of the urine. In one instance the indican reaction was also increased.

naphthaquinone compound forms the basis of the method of separation, the outlines of which are given above.

¹ A good discussion of Ehrlich's dimethylamidobenzaldehyde reaction in the urine by C. E. Simon may be found in the *American Journal of the Medical Sciences*, cxxvi, p. 471, 1903. Bauer (*Zentralblatt für Innere Medizin*, 1905, No. 34, p. 833) brings forward evidence to support the view that the Ehrlich reaction depends on urobilogen. This view appears to me not irreconcilable with the experimental results obtained by me.

A METHOD FOR THE QUANTITATIVE DETERMINATION OF INDOL.

BY C. A. HERTER AND M. LOUISE FOSTER.

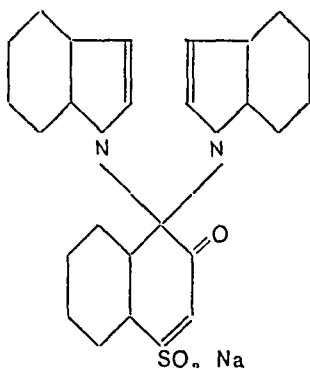
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The condensation of indol with β -naphthaquinone-sodium-monosulphonate, a color-reaction at once sensitive and striking, has been used by us as the basis of a method for the quantitative estimation of indol. In a previous paper¹ by one of us, a description of this reaction is given. A dilute solution of indol (say 1:100,000 parts water), made slightly alkaline with potassium hydroxide, gives with one drop of 2 per cent. naphthaquinone-sodium-monosulphonate a blue or green-blue color. This color is varied by excess of either reagent and by heat. If to a warm, more concentrated solution of indol the alkali is added and then the naphthaquinone compound, a dark precipitate is formed, which upon examination will be found to consist of well-defined acicular needles, bluish in color, and closely felted together. This compound is very slightly soluble in water, more soluble in alkali, and moderately soluble in chloroform, with the production of a red color.

Analysis to determine the nitrogen content of this substance showed the presence of two nitrogen atoms in the molecule, indicating that the compound is derived from two molecules of indol, while the determination of the sulphur content made it evident, first, that the sulphonic acid group is retained, and secondly, that but a single molecule of β -naphthaquinone-sodium-monosulphonate enters into the combination. The condensation probably takes place, therefore, between one of the carbonyl groups of the naphthaquinone compound and the imide groups of the indol molecules, with the elimination of water. It is probable that the indol substituents occupy the para position to the sulphonic acid group, and that the constitution of the

¹ *Jour. of Exper. Med.*, vii, No. 1, 1905.

compound, di-indyl-di-hydronaphthaline-keto-sodium-monosulphonate may be represented thus:



It must be admitted, however, that other constitutions for this compound are imaginable, and it must not be forgotten that the pyrrol ring may be broken at the double bond.

A slight blue precipitate forms in solutions containing one part of indol to 256,000 parts of water; in greater dilution the coloration is green, and fails entirely when the dilution is 1:1,024,000 parts. Chloroform indicates the presence of di-indyl-di-hydronaphthaline-keto-sodium-monosulphonate by its faintly pink color, even in this extreme dilution. The sensitiveness of the di-indyl-di-hydronaphthaline-keto-mono-sulpho-nate reaction, the insolubility of the newly formed compound in water, and the thorough extracting power of chloroform suggested the possibility of a quantitative method for the determination of indol and experiments were made with this idea in view.

Some carefully prepared di-indyl-di-hydronaphthaline-keto-sodium-monosulphonate was washed and dried to constant weight, placed in a separatory funnel in which was a small quantity of water, and extracted with chloroform in small portions until the chloroform showed no coloration. The combined portions of chloroform were then distilled, the residue transferred to a tared watch-glass, the last traces of chloroform allowed to evaporate, and the weight obtained. It was found that essentially the entire quantity of the di-indyl compound could be recovered.

The condensation of indol with the naphthaquinone com-

pound requires even in dilute solutions considerable time for its completion, and it has been found desirable to allow a period of not less than ten minutes to elapse before shaking out the condensation product by means of chloroform. If the product be shaken out with chloroform before the reaction has been completed, the uncombined indol passes into the chloroform and a loss is thus occasioned. It is also important to add a slight excess of the naphthaquinone compound in order to secure the condensation of all the indol present. The naphthaquinone solution should be added in an amount sufficient to give the solution a distinctly yellow tinge after the condensation product has been shaken out with chloroform, this tinge being an evidence of the presence of the naphthaquinone compound in excess.

Another experiment was performed with the components of the above compound. A weighed portion of indol, 0.02 gram, was transferred to a separatory funnel and dissolved in the smallest quantity of water possible, made alkaline with potassium hydroxide, and the naphthaquinone compound added in slight excess. The characteristic precipitate which formed was then shaken with chloroform until the latter remained uncolored, the chloroform distilled, and the residue cooled and weighed. Of the original indol, 96.5 per cent. were recovered. Further experiments were made to ascertain whether the indol could be recovered from a solution containing a proteid. A 0.3 per cent. bouillon, made from beef extract and Witte's peptone, was selected for this purpose. A weighed portion (0.02 gram) of indol was dissolved in the smallest quantity of water and added to about 150 c.c. of the bouillon and the solution made slightly alkaline and distilled. The last portion of the distillate was tested and found still to contain traces of indol; 100 c.c. of water were added and the process continued until negative results were obtained on testing a few drops of the distillate with both the naphthaquinone reagent and with para-dimethylamidobenzaldehyde. The distillate was then made alkaline, the indol precipitated with β -naphthaquinone-sodium-monosulphonate, and the di-indyl compound extracted with chloroform. Good results were obtained, even with a slightly acid bouillon.

A similar experiment was made with Dunham's peptone solution, and equally good results were obtained, 97.3 per cent. being the proportion of indol recovered.

Some observations have been made which indicate that the method here described will prove of service in determining the indol content of the feces. The material to be examined is rendered alkaline with caustic potash in order to hold back phenol. Distillation, preferably with steam, is then practiced. The distillate contains ammonia and perhaps indol and skatol. In order to remove the ammonia, which gives a green color with the naphthaquinone compound, it is necessary to acidify the distillate and distill again. This distillate contains the indol and skatol. The indol is separated from the solution by adding to it an excess of the naphthaquinone compound and then alkalizing with caustic potash. Under these conditions the blue condensation product of indol and naphthaquinone is gradually formed and can be almost completely shaken out by chloroform. A large excess of the naphthaquinone compound should be avoided, as this substance passes to a slight extent into the chloroform and may thus slightly modify the red color imparted to it by the indol compound.

It has been easy to show by means of this method that there is a rough parallelism between the intensity of the indican reaction of the urine and the quantity of indol in the feces.

If the quantity of indol to be determined is very small (say less than 0.25 mg.), it is necessary to concentrate the red compound by evaporation, and in any case one should employ for the colorimetric determination of indol a distinctly pink solution of the di-indyl compound in chloroform. The quantity of indol present may now be estimated colorimetrically, preferably by means of the Duboscq colorimeter. It has been found that a solution of Casella & Co.'s brilliant cochineal can be used for comparison, as it gives tints closely resembling those of the indol compound in chloroform, but it is preferable to use as a standard of comparison a chloroform solution of the indol condensation product kept for this purpose or made up freshly. For this purpose 0.5 milligram of indol may be employed and its compound dissolved with 10 or 15 c.c. of chloroform. The chloroform solutions to be compared are then placed in the cups

belonging to the Duboscq instrument. By means of this instrument it is possible to obtain fairly close correspondences between solutions of the condensation product prepared from equal quantities of indol. We have thus a standard for comparison with the condensation product obtained from the indol distilled from the fæces or other putrefactive material. It is believed that this method will prove more accurate than any now in use for the quantitative determination of indol and it can be recommended for simplicity.

The reagent with which the condensation with indol is brought about is at present difficult to obtain in this country, but it is our intention to request the leading dealer in chemicals in New York to import it and keep it in stock. Dr. T. Schuchardt of Görlitz, Germany, has recently listed the ammonium salt of the naphthaquinone compound, which one would expect to answer the requirements as well as the sodium salt.

THE CUTANEOUS EXCRETION OF NITROGENOUS MATERIAL.

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While the larger amount of the nitrogen excreted from the body is eliminated in the urine, in the form of urea, uric acid, creatinin, and allied compounds, nitrogenous material may also leave the body in the feces and perspiration. There is indeed a possibility of the excretion of free nitrogen in the respiratory and intestinal gases, for, unfortunately, although most physiologists assume that no such excretion of nitrogen takes place, a fundamental demonstration of the correctness of this assumption is lacking. Usually, however, it is considered that the total output of nitrogen is that in the urine and feces. It has long been known that nitrogenous compounds are excreted through the skin, but it has commonly been accepted that the amount thus excreted is extremely small—in fact, too minute to take into consideration in ordinary metabolism experiments.

An examination of the literature of the subject shows that widely varying results have been obtained. Thus Favre¹ found 0.044 gram of urea per 1000 c.c. of perspiration, corresponding to about 0.02 gram of nitrogen, while Funke² found a much larger amount, namely, 1.55 grams of urea in 1000 c.c. of perspiration, corresponding to 0.87 gram of nitrogen. Argutinsky³ found 0.363 and 0.410 gram of urea in 225 and 330 c.c. of perspiration respectively. By extracting with distilled water the clothes worn by subjects actively walking or doing other vigorous exercise during a considerable portion of the day, he also found

¹ *Compt. rend. Acad. d. Sc., Paris*, xxxv, p. 721, 1852.

² *Untersuch. z. Naturl. d. Mensch. u. d. Thiere*, iv, p. 36, 1858.

³ *Arch. f. d. ges. Physiol.*, xlv, p. 600, 1890.

0.7 gram of nitrogen. Esterbrook,¹ in some experiments upon himself, obtained from 0.1 to 0.3 per cent. urea in the perspiration.

The most complete list of investigations into the subject of human perspiration that is accessible to the writer is that of Hoelscher,² published in connection with the description of his elaborate series of experiments, though it is regrettable that the references to the places of publication of all the earlier work are omitted in this valuable experimental contribution to the subject. In studying human perspiration under varying conditions, inducing perspiration by hot-air baths, he found as the average of twenty-two experiments in which 6719 c.c. of perspiration were collected, that 1000 c.c. of perspiration contained 7.1 grams of solids, of which about 0.6 gram was urea and the total nitrogen content 0.48 gram.

W. Camerer³ found in experiments in which perspiration was induced by the electric light, hot air, and vapor bath, that three 100 c.c. samples of perspiration contained respectively 0.169, 0.137, and 0.091 gram of nitrogen.

That there is an appreciable amount of nitrogenous material excreted through the skin even of an infant is shown by the experiments of Rubner and Heubner⁴ and Tangl.⁵ The former investigators found 2.83 milligrams of ammonia and 0.0205 milligram of urea per day, and estimated the total nitrogen output through the skin to be 39 milligrams per day. Tangl reported an average of 44 milligrams per day.

Perhaps the largest recorded amount of nitrogen found in perspiration not induced by muscular work was that obtained in experiments reported by Eijkmann⁶ in three experiments with Malay medical students in Java. He obtained 0.222 gram of nitrogen in a three-hour experiment, and in two twenty-four-hour experiments, 0.761 gram and 1.362 grams respectively. The subjects were engaged in light occupation, and the perspiration was induced by the tropical climate of Java.

¹ *Scottish Med. and Surg. Journ.*, vi, pp. 120-140, 1900.

² *Journ. Amer. Med. Assoc.*, 1899, June 17, pp. 1-16.

³ *Zeitschr. f. Biol.*, xli, p. 271, 1901.

⁴ *Ibid.*, xxxvi, p. 34, 1898.

⁵ *Arch. f. d. ges. Physiol.*, civ, p. 483, 1904.

⁶ *Virchow's Arch. f. path. Anat. u. Physiol.*, cxxxi, p. 170, 1893.

When perspiration is induced by severe muscular exercise the elimination of nitrogenous material may be very large.

In experiments in this laboratory with the respiration calorimeter,¹ the subjects of certain experiments devoted a considerable part of the day, i. e., eight hours, to work upon a stationary bicycle. The amount of nitrogen found in the clothes by extraction with distilled water varied from 0.2 to 0.66 gram per day, the average of eighty-eight days being 0.29 gram.

In 1900, during a study of the food consumed and digested by four members of the Harvard University boat crew,² there was apparently a large retention of nitrogen in the body. It was there pointed out that probably a not inconsiderable portion of the observed gain was to be accounted for by the fact that there might be a large loss of nitrogen in the profuse perspiration resulting from the very severe muscular exercise attendant upon training for a Varsity boat-race.

In a recent study of metabolism with athletes, Lavonius³ found in one case 0.14 per cent., and in the other 0.9 per cent. nitrogen in the perspiration. Using the minimum figure and assuming from measurements of loss of body weight that the weight of perspiration is about 1.8 kilograms, he calculates the loss of nitrogen to be 1.8 grams per day in a circus athlete.

It is thus evident that especially under conditions which result in profuse perspiration, such as tropical climate, or excessive muscular exercise, a not inconsiderable excretion of nitrogenous material through the perspiration may take place, and further knowledge regarding the amount of nitrogenous material thus excreted is much needed. Furthermore, it is evident that data regarding the excretion of nitrogen under ordinary conditions where there is no sensible perspiration would be of considerable value. In connection with the series of metabolism experiments which are continually in progress in this laboratory, opportunity was had to observe in a number of cases the

¹ U. S. Dept. Agr., Office Expt. Sta. Bull. 136, p. 118, 1903.

² Atwater and Benedict, *Boston Med. and Surg. Journ.*, cxliv, p. 634 1901.

³ *Skand. Arch. f. Physiol.*, xvii, p. 196, 1905.

excretion of nitrogenous material through the skin under conditions of both rest and severe muscular work.

METHOD.

Before the metabolism experiment began, the subject took a good scrubbing without using soap. This was followed by a shower-bath, and finally the whole body was carefully sponged with clean cheese-cloth and distilled water. A union suit of cotton and cotton stockings were previously thoroughly washed and extracted with distilled water, and after thoroughly drying the body they were put on. At the end of the experiment the union suit and socks were removed and the body carefully sponged with distilled water, all the wash water being carefully saved. The union suit and stockings were then extracted with distilled water several times (never less than four and frequently eight). The water was made slightly acid to prevent any escape of ammonia during evaporation and the whole mass of wash water concentrated into a small bulk. It was then filtered and only the clear filtrate evaporated, thus eliminating completely epithelial scales, hair, fragments of clothing, or other dust. In consequence none but water-soluble nitrogenous compounds are here considered. On evaporation, the liquid frequently was turbid and before final analysis it was filtered, the nitrogen in the filtrate in certain cases being determined separately from the nitrogen in the precipitate.

The samples were subjected to the usual Kjeldahl process for the determination of nitrogen.

As a test of the care with which the union suits were extracted before use, a previously extracted suit was again extracted, the water used for washing and extracting the suit evaporated, and the nitrogen determined as above described. The amount of nitrogen thus found as a result of re-extracting the new union suit was but 25 milligrams.

My thanks are due to Mr. Ernest M. Swett, formerly assistant in this laboratory, for the painstaking care with which he carried out his important share in this investigation.

The experiments here reported consist of two kinds: first, those when the subjects were at rest; and, second, those when the subjects were at severe muscular work.

REST EXPERIMENTS.

I. Experiment with L. L. A. (Dec. 16-22, 1904):

The subject of this experiment, a man 24 years old and weighing without clothing 74 kilograms, remained in the respiration chamber 7 days, from December 16-22, of which the first 4 days were without food and the last 3 days with food. During this period he was at rest and, indeed, the routine of life was such as to call for much less muscular activity than that to which he was ordinarily accustomed. Practically all of the waking hours were devoted to sitting in a chair and reading or writing. The subject wore the union suit and stockings continuously from the beginning to the end of the experiment. After leaving the chamber, the water used in extracting the clothing and in sponging the body contained 0.722 gram of nitrogen, or since the experiment lasted 7 days, 0.103 gram per day.

II.—Experiment with B. A. S. (Jan. 7-11, 1905):

During this experiment the subject, a man 23 years old and weighing without clothing 62 kilograms, remained inside the respiration chamber 5 days, of which 4 days were without food and the last day with food. Save for a ten-minute period on the first day, when exercise was taken on a bicycle ergometer inside the respiration chamber, the muscular exercise was not unlike that of the experiment with L. L. A., very much less than that to which the subject was ordinarily accustomed. After the termination of the experiment, the water from the bath was found to contain 0.0854 gram of nitrogen and the water used in extracting the clothing 0.0599 gram of nitrogen. Since this experiment lasted 5 days, the total excretion of nitrogen measured here amounts to 0.029 gram per day.

III.—Experiment with B. A. S. (Jan. 28-Feb. 4, 1905):

During this experiment the subject remained in the respiration chamber 8 days, of which 5 days were without food and 3 days were with food. The muscular activity was about that of the other experiment with B. A. S., save that even the ten-minute exercise period of the first day of the former experiment was not repeated. The nitrogen found in the bath water from this experiment amounted to 0.3822 gram, and that in the water used for extracting the clothing 0.2659 gram, a total of 0.6481 gram. As the experiment lasted 8 days, this is an average of 0.081 gram per day.

IV.—Experiment with B. A. S. (Mar. 4-13, 1905):

The subject remained in the chamber for 10 days, of which 7 days were without food, followed by 3 days with food. The muscular activity closely approached that of Experiment III. The nitrogen content of the bath water was 0.162 gram and the water used to extract the clothing contained 0.375 gram, thus the total measured nitrogen elimination in the perspiration amounted to 0.537 gram. Since the experiment lasted 10 days, the amount per day was 0.0547 gram.

V —Experiment with B. A. S. (April 8-11, 1905):

The experiment lasted 4 days, the conditions being exactly those of Experiment IV so far as muscular activity was concerned, except that during the entire period the subject fasted, consequently the muscular exertion involved in receiving and eating the food was not a factor. At the end of the experiment the water from the bath contained 0.152 gram of nitrogen, and the extract from the clothing contained 0.194 gram nitrogen. The total nitrogen elimination for 4 days was therefore 0.346 gram or 0.087 gram per day.

CONCLUSIONS REGARDING NITROGEN EXCRETION DURING REST.

As a result of the data given above, it is seen that even when the subject has no muscular exercise there is a measurable quantity of nitrogenous material excreted through the skin each day. While there is considerable variation in the actual quantity thus determined, the average is 0.071 gram per day.

The exact nature of the nitrogenous material thus excreted was not studied. That it is in large measure urea or ammonium compounds is highly probable, though the presence of soluble proteids is not at all impossible. Since in some cases at least the perspiration is alkaline and there would be a tendency to lose ammonia by gradual decomposition, these figures must be looked upon as representing the minimum rather than the maximum amounts, and the fact remains that the amount thus excreted per day is certainly worthy of consideration in metabolism experiments, especially where small quantities of nitrogen in the intake and output are involved.

Zuntz¹ has already noted this fact and estimates that the loss of nitrogen due to perspiration, wearing off of epidermis, etc., amounts to 0.46 gram of nitrogen per day. What proportion of this loss is due to perspiration alone he does not state.

WORK EXPERIMENTS.

Aside from the earlier published data regarding the experiments with work carried out in this laboratory, opportunity was had to study the nitrogenous excretion of a professional athlete (B. N.) during experiments of short duration in which very violent muscular exercise was taken. The experiments lasted in general not more than 3 or 4 hours. The routine and technique were practically identical with those given above for Experiments I-V.

In evaporating the filtered wash- and extraction-water, a precipitate

¹ *Ber. d. deutsch. pharm. Gesellsch.*, xii, p. 363, 1902.

frequently formed. An attempt was made in some experiments to determine the nitrogen in this precipitate as well as in the concentrated wash water.

VI.—Experiment with B. N. (Jan. 23, 1905):

During this experiment the subject, a professional bicyclist, 28 years old and weighing without clothing 62 kilograms, rode a bicycle ergometer in the respiration calorimeter for a period of 4 hours. The muscular exercise was very severe, as was evidenced by the fact that the subject left the chamber in a profuse perspiration and the union suit and stockings were soaked with perspiration. The amount of muscular work performed may be seen from the fact that the total output of heat for this experiment was nearly 600 calories per hour. The bath water gave in the filtrate 0.063 gram of nitrogen, and in the precipitate 0.0037 gram of nitrogen. The extract water from the clothing gave in the filtrate 0.785 gram and in the precipitate 0.018 gram.

The total output of nitrogen, therefore, during this four-hour experiment was 0.87 gram or 0.22 gram per hour.

VII.—Experiment with B. N. (Jan. 24, 1905):

For 5 hours the subject of this experiment rode the bicycle ergometer inside the respiration chamber. The resistance of the ergometer was made somewhat less than in Experiment VI, though the experimental period was an hour longer. The clothing was thoroughly wet through with the perspiration. The bath water contained 0.145 gram of nitrogen, while the extract water contained 0.682 gram. The total output of nitrogen as measured therefore was 0.827 gram for the five-hour experiment, or 0.165 gram per hour.

VIII.—Experiment with B. N. (Jan. 25, 1905):

A second five-hour experiment was made in which the resistance of the bicycle ergometer was still more diminished, thus decreasing materially the total amount of work done. The perspiration, while profuse, seemed to the subject to be somewhat less than in Experiment VI, though the clothing at the conclusion of the experiment appeared about as wet as in the earlier experiment. The bath water from this experiment contained 0.13 gram of nitrogen, while the extract from the clothing contained 0.45 gram of nitrogen. The total output for the five-hour experiment was 0.58 gram or 0.11 gram per hour.

IX.—Experiment with B. N. (Jan. 26, 1905):

The resistance of the bicycle ergometer was still further decreased in this experiment, and the work performed accordingly reduced to not far from one half that done in Experiment VI. The experiment lasted only 3 hours, as the subject was obliged to leave Middletown to meet a professional engagement. We were unable to extract the underclothes used in this experiment, as the subject wished to take them with him for use in an approaching race. The bath water contained 0.10 gram of nitrogen, however, and if we examine the results in Experiments VII and VIII and note the proportion of nitrogen contained in the extract water when compared to that in the bath water, we see that in general the extract

water contained not far from three to four times that in the bath water. Using the minimum figure in this case it is estimated that 0.3 gram would have been found in the extract water had the clothing been extracted. The total output for the three-hour experiment was therefore probably not far from 0.4 gram or 0.13 gram per hour.

CONCLUSIONS REGARDING NITROGEN EXCRETION DURING WORK.

The increase in the amount of nitrogen-containing material excreted through the skin when the subject is engaged in severe muscular labor is markedly noticeable, for while during rest-experiments the amount of nitrogen thus excreted is about 0.071 gram per day, hard muscular labor may result in an excretion equivalent to 0.22 gram of nitrogen in one hour. Furthermore, the results show with considerable regularity a nitrogenous excretion roughly proportional to the amount of work done: varying in these experiments from 0.22 gram per hour in Experiment VI, when the most severe work was performed, to 0.13 gram per hour in Experiment IX, in which the work done was less by about one half.

Of greatest significance is the important bearing of this channel for the excretion of nitrogenous material in experiments on the metabolism of proteid. Profuse perspiration, whether induced passively or by muscular work, results in a considerable excretion of nitrogenous material through the skin. While the work engaged in during these experiments was severe, certainly that of Experiment IX was not extraordinarily so, and might well be equalled by many men engaged in occupations involving muscular work. A total excretion equivalent to one or more grams of nitrogen per day is not at all inconsiderable, and hence in accurate metabolism experiments we must give recognition to the possibility of excretion through this hitherto almost unconsidered channel. Especially is this so in experiments where the total amounts of nitrogen in the ingesta and egesta are smaller than normal, since the percentage error is thereby proportionally increased.

THE FORMATION OF GLYOXYLIC ACID.

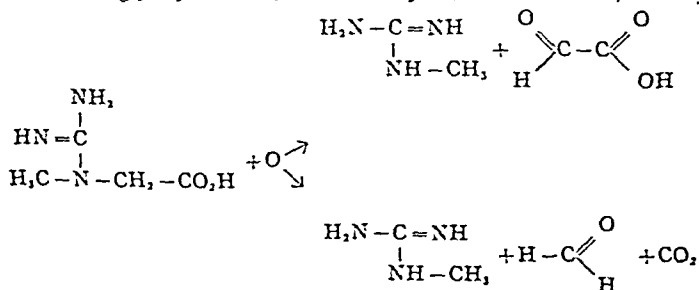
By H. D. DAKIN.

(From the Laboratory of Dr. C. A. Herter.)

(Received for publication, January 22, 1906.)

In a recent paper¹ it was shown that certain amino-acids readily undergo oxidation at the ordinary temperature when treated with hydrogen peroxide and a trace of ferrous sulphate. The products of the reaction were found to be aldehydes and acids containing one less carbon atom than the parent acid, together with ammonia and carbon dioxide. In the case of glycocoll, it was found that glyoxylic acid was formed in addition to the other oxidation products, namely formaldehyde, formic acid, ammonia, and carbon dioxide. It was therefore of interest to inquire whether glyoxylic acid did not occur in the animal body as an intermediate product of metabolism. A paper upon this subject has already appeared by Eppinger² to which reference will be made later.

It was obviously a question of importance to know what substances in addition to glycocoll yield glyoxylic acid as a result of oxidative changes. The first substances to be tried were creatin and creatinin. Both of these substances are readily oxidized by hydrogen peroxide and ferrous sulphate and yield as products, *glyoxylic acid*, formaldehyde, formic acid, methyl-



¹ This Journal, i, p. 171, 1906.

² Eppinger, *Beitr. z. chem. Physiol. u. Pathol.*, vi, p. 492, 1905.

guanidin, and carbon dioxide. The larger portion of the formaldehyde is converted into formic acid by the further action of a part of the hydrogen peroxide. The changes are exactly similar in the case of creatinin. The formation of methylguanidin from creatin on boiling with mercuric oxide was long ago observed by Dessaignes¹ and a similar reaction occurs with creatinin when treated with potassium permanganate.² In an actual experiment creatinin was dissolved in water and a small excess over the calculated amount of hydrogen peroxide solution added together with a trace of ferrous sulphate. The peroxide solution was allowed to stand over night and then distilled. A small amount of formaldehyde was found in the distillate and could be detected by means of the Lebbin and other reactions even when only a few milligrams of creatinin were oxidized. On acidifying with sulphuric acid and redistilling, the distillate was found to contain formic and glyoxylic acids,³ the latter substance giving an intense reaction when treated with tryptophan or indol or skatol and concentrated sulphuric acid.⁴ The residue was freed from sulphuric acid by means of barium carbonate and the filtrate acidified with hydrochloric acid and then concentrated to small bulk on the water bath. On precipitation with gold chloride solution, beautiful shining yellow crystals of the gold salt of methylguanidin were easily obtained.

From the results already obtained it was considered probable that other substituted acetic acids would yield glyoxylic acid upon oxidation and accordingly glycollic acid, sarcosin, betain, and hippuric acid were treated with hydrogen peroxide and ferrous sulphate in the same way as glycocoll and creatin.

Under the special conditions of the experiment the first two acids are readily oxidized but yield only minute traces of glyoxylic acid, while, on the other hand, betain and hippuric acid

¹ Dessaignes, *Liebig's Ann. d. Chem.*, xcii, p. 407, and xcvii, p. 340.

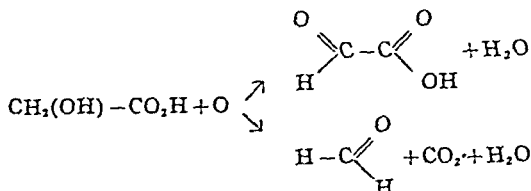
² Neubauer, *ibid.*, cxix, p. 46.

³ The formation of glyoxylic acid may be found to be a useful confirmatory test for the presence of creatin, creatinin, or glycocoll. The reaction is delicate and easily carried out, but it is necessary to avoid the presence of other substances which yield traces of the acid.

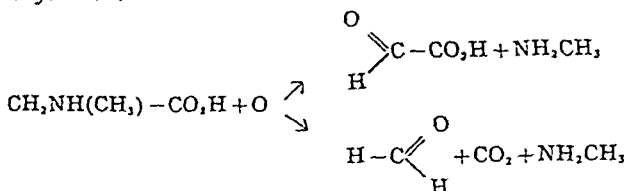
⁴ Hopkins and Cole, *Proc. Roy. Soc.*, lxxviii, p. 21, 1901.

are attacked with the greatest difficulty but, notwithstanding, they yield definite traces of glyoxylic acid.

Glycollic acid on oxidation with the peroxide yields mainly formaldehyde, the larger part of which is further oxidized to formic acid:



Sarcosin in similar fashion yields the same products together with methylamin:



In each case the formic acid was identified as the lead salt while the methylamin was converted into the platinum salt. It should be noted that formic acid interferes considerably with the color reactions for the detection of glyoxylic acid and should be removed as completely as possible.

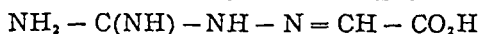
In the case of the oxidation of hippuric acid and betain, the bulk of the substance was recovered unchanged at the end of the experiment, but the formation of small quantities of glyoxylic acid was readily demonstrated.

It is of interest to note too that a little glyoxylic acid is also formed when dextrose or peptone is treated with excess of hydrogen peroxide and a trace of ferrous sulphate.

From the foregoing results it is clear that there are a number of substances of biochemical importance, in addition to those already known, which by gentle oxidation at low temperatures yield glyoxylic acid, and it was therefore of interest to try to detect the acid among the products of metabolism.

The isolation of traces of glyoxylic acid in complicated mixtures such as tissue extracts is by no means easy. In pure

solutions, the usual methods made use of have depended upon the formation of the calcium salt or of the phenylhydrazone of the acid, while qualitatively the anilin oxalate test described by Perkin and Duppa¹ and especially the reactions with skatol, indol, and tryptophan described by Hopkins have been chiefly employed. The phenylhydrazone of glyoxylic acid is not very well suited for the identification of small quantities of the acid, and preliminary experiments have shown that a more satisfactory method may be found in the formation of a very sparingly soluble crystalline substance obtained by acting upon glyoxylic acid with amidoguanidin. The substance crystallizes from water in beautiful needles which contain water of crystallization and which are very soluble in both acids and alkalies. It is apparently identical with the substance obtained by Thiele and Dralle² by heating chloralamidoguanidin with water, and has also been obtained by Doebner.³ It has the formula:



Its melting-point is not very sharp, but after drying at 100°C to remove water of crystallization it melts at about 155°C.

It may be said at once that by the employment of one or other of the above methods, it has been possible to obtain evidence of the existence of glyoxylic acid in animal tissues and secretions, and also in media of various kinds in which bacteria or mould have grown. For reasons which will be mentioned later, it is not, however, certain that the glyoxylic acid is produced in all cases by the organism. Eppinger⁴ has recently employed the reaction with indol for the detection of glyoxylic acid in urine and has carried out the test in many cases by direct addition of strong sulphuric acid to 3-5 c.c. of the urine which has previously been mixed with an indol solution. A red color was supposed to indicate the presence of glyoxylic acid. It is certain that this method is useless for the purpose for which it was employed. The presence of nitrates and nitrites in the urine, which was long ago recognized by Wolffius⁵ and by Schönbein,⁶ must obviously

¹ Perkin and Duppa, *Ber. d. deutsch. chem. Gesellsch.*, xix, p. 595.

² *Liebig's Ann. d. Chem.*, cccii, p. 279.

³ *Ibid.*, cccxv, p. 1, 1901.

⁴ Eppinger, *Beitr. z. chem. Physiol. u. Path.*, vi, p. 492, 1905.

⁵ Wolffius, *Diss.*, Dorpat, 1861.

⁶ Schönbein, *Journ. f. prakt. Chem.*, xcii, p. 156; xciii, 463.

interfere with the test, since the red color of nitroso-indol would simulate that possibly produced by glyoxylic acid. In addition, the action of strong sulphuric acid upon undiluted urine gives rise to color changes which render almost impossible the observation of any effect due to the presence of minute quantities of glyoxylic acid. Eppinger makes the statement that skatol cannot be substituted for indol in the direct testing of urines for glyoxylic acid, and suggests that substances must be present in the urine which prevent the reaction. As a matter of fact, however, the skatol reaction is even more sensitive than that with indol, and the observation of Eppinger serves only to show that the red colors obtained by him in the direct testing of urines were simply due to nitroso-indol and not to glyoxylic acid.¹ Eppinger also tried to obtain glyoxylic acid in the distillate from urines acidified with phosphoric acid, but states² that its separation in quantity by this means was not successful, owing, it is suggested, to the affinity of the glyoxylic acid for urea or other unknown substances. This explanation, however, is hardly satisfactory, for, if the glyoxylic acid undergoes condensation with urea, the presence of the resulting product—allantoin—can no longer be regarded as evidence of the occurrence of glyoxylic acid in the urine; while, on the other hand, urea salts of volatile acids are extremely readily hydrolyzed even by water, and no difficulty would be found in detecting the acid in the distillate.

As a result of many experiments, it has been found that it is possible to obtain a substance from urine which gives many of the reactions for glyoxylic acid, but the quantity is extremely small. If fresh urine or an extract of liver, muscle, or blood which has been roughly freed from proteid³ be distilled with a little phosphoric acid,⁴ a weak acid distillate is obtained which often gives the skatol and indol tests directly. Experiments were made to determine the best method of concentrating very weak glyoxylic-acid solutions. Concentration of the free acid on the water-bath was obviously impossible, as was also the use

¹ Cf. Inada, *Beitr. z. chem. Physiol. u. Path.*, vii, p. 473, 1905.

² *Loc. cit.*, p. 496.

³ Acetic acid must not be used for the coagulation.

⁴ Sulphuric acid should not be used, as even comparatively pure acid may give enough nitrous acid to interfere with the reaction.

of the sodium or potassium salts. Addition of excess of ammonia and subsequent evaporation resulted in great loss of acid, which was not completely avoided by evaporation *in vacuo* at 45° C. The method eventually used was to add small quantities of calcium bicarbonate solution to the distillate and then to slowly concentrate. From most tissues a solution was readily obtained which gives not only the skatol and indol reaction but also the reaction with tryptophan.¹ The reaction with tryptophan is not so delicate as the skatol test,² but I believe that a positive result with this test is of more value than any of the other color reactions.

Assuming that traces of glyoxylic acid do occur in the urine, it is of interest to know what significance can be attached to the fact. Eppinger has suggested that the presence of glyoxylic acid may be of clinical significance and has commonly obtained positive results in cases of dysentery, typhoid, and also after alcohol consumption. Although it is clear that glyoxylic acid may be regarded as an intermediate product of metabolism the origin of these traces of glyoxylic acid is by no means clear. In the first place, as has already been shown, creatin and creatinin readily yield glyoxylic acid on oxidation with hydrogen peroxide. Schönbein³ in 1864 was able to detect hydrogen peroxide in the urine and it is therefore open for one to account for the presence of glyoxylic acid in the urine in a much more simple way than that suggested by Eppinger, namely by the simple oxidation of creatinin by oxidizing agents present in the urine. What is true for creatinin also holds for creatin, glycocoll, hippuric acid, alcohol, etc. If a normal urine be taken and hydrogen peroxide be added, with or without the addition of a catalyst, considerable quantities of glyoxylic acid are readily formed. The glyoxylic acid is apparently derived mainly from the creatinin, and possibly glycocoll, since hippuric acid, peptone, or sugar yield relatively much smaller quantities.

¹ The tryptophan for these experiments was kindly furnished by Miss M. L. Foster who prepared it by the tryptic digestion of casein.

² The color reaction between skatol and glyoxylic acid is still perceptible even when the solution (2-3 c.c.) contains only one hundredth of a milligram of acid. The sulphuric acid used should be as free as possible from oxides of nitrogen.

³ Schönbein, *Journ. f. prakt. Chem.*, xcii, p. 168, 1864.

Another point which seems to have been insufficiently considered is the fact that many substances readily undergo auto-oxidation with the production of traces of glyoxylic acid. The fact that acetic acid commonly contains glyoxylic acid has been made clear by Hopkins, and many chemicals in an ordinary state of purity, such as lactic acid, acetone, alcohol, etc., show traces of glyoxylic acid when carefully tested. In the course of making controls in experiments in which organisms were grown upon media containing glycocoll and creatinin together with inorganic salts, I have observed that the solutions prepared from substances containing no glyoxylic acid, when sterilized and preserved under aerobic conditions, quickly yield traces of a substance which is not to be distinguished from glyoxylic acid by any of the reactions suitable for the detection of minute quantities.

For example, nutrient media containing about 0.5 per cent. of glycocoll or creatinin, together with traces of inorganic salts (sodium chloride, magnesium sulphate, calcium chloride, and potassium phosphate), although remaining perfectly sterile gave strong reactions for glyoxylic acid shortly after their preparation.¹

Subsequently it was found that the formation of glyoxylic acid from glycocoll was readily shown if a solution of glycocoll was simply evaporated on the water-bath, with a little calcium bicarbonate solution to prevent loss of free acid. On acidifying and distilling the residue from such an experiment there is no difficulty in detecting glyoxylic acid in the distillate.

From these results it is concluded that it still is an open question as to how far one is justified in assuming the previous existence of glyoxylic acid in tissues which have been subjected to chemical manipulations which might easily result in the production of glyoxylic acid as a result of the auto-oxidation of other substances normally present in the tissues. It is clear that the experimental difficulties of deciding the question are considerable, for since glyoxylic acid is not only a very reactive substance but is also a comparatively strong poison, one would not expect a large quantity of such a substance to be present at any particular time. The idea of the formation of an aldehyde acid as an in-

¹ Further experiments upon the auto-oxidation of amido-acids will be undertaken. Some preliminary results indicate that the action of light is of influence in the process.

intermediate product of metabolism is in many respects attractive and it is possible that further experiments will enable us to reach a definite decision.

In conclusion I wish to express my thanks to Professor Herter, to whom I am indebted for the opportunity of carrying out the foregoing work and from whom I have received much helpful criticism.

CONCLUSIONS.

1. Creatin and creatinin readily yield large quantities of glyoxylic acid on oxidation with hydrogen peroxide. Smaller amounts are also obtainable from glycollic acid, sarcosin, betain, and hippuric acid.

2. A substance giving the reaction of glyoxylic acid is readily formed by the auto-oxidation of aqueous solutions of glycocoll, creatin, and creatinin.

3. Although small quantities of glyoxylic acid may be isolated from blood, liver, and muscle, from urine, and from culture media in which bacteria or moulds have grown, it is not certain that it is a direct product of cell metabolism.

4. The test proposed by Eppinger for the detection of glyoxylic acid in urine is unreliable.

5. The presence of traces of glyoxylic acid in the urine may be accounted for by the oxidation of creatinin. Addition of hydrogen peroxide to urine gives rise to the formation of a considerable quantity of glyoxylic acid.

STUDIES IN THE CHEMISTRY OF THE ION-PROTEID COMPOUNDS.

(Second Communication.¹)

II. ON THE INFLUENCE OF ELECTROLYTES UPON THE STAINING OF TISSUES BY IODINE-EOSIN AND BY METHYL GREEN.

By T. BRAILSFORD ROBERTSON.

(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, December 20, 1905.)

I. INTRODUCTORY.

In 1896 Kahlénberg and True² suggested that the toxic effects of electrolytes in dilute aqueous solution might be due to the action of the *ions* into which the electrolyte is dissociated and they brought forward experiments in support of their hypothesis. The proofs were not entirely satisfactory, however, but in 1897 Loeb published a paper³ in which he demonstrated that the effects of strong inorganic acids on absorption of water by muscles could be referred to the hydron, which is the characteristic and predominant ion in acids, inasmuch as equal quantities of hydron in solutions of these acids produced quantitatively equal effects. The same principles were shown to hold good for alkalies—in this case the hydroxyl ion being predominant. These investigations placed the whole matter of the action of electrolytes on living tissues in a new light—for the physiological action of acids and bases was then shown to run parallel with their other properties, such as electrical conductivity, "avidity," etc., which were known to be primarily determined by the H⁺.

¹ The first of these studies, "Ueber den Einfluss von Elektrolyten auf die Frequenz des Herzschlag," appeared in *Pflüger's Arch. f. d. ges. Physiol.*, cx, p. 610, 1905.

² *Botan. Gaz.*, xxii, p. 81, 1896.

³ "Physiologische Untersuchungen über Ionenwirkungen, I," *Pflüger's Arch. f. d. ges. Physiol.*, lxi, p. 1, 1897.

and OH' ions respectively. The inference was irresistible that the physiological action also depended on these ions. Subsequent investigations,¹ however, showed that the observed physiological phenomena could not be entirely accounted for on a purely physical basis, and in 1899 Loeb suggested the hypothesis that the ions which entered a muscle must alter its substance chemically.² In a subsequent paper³ he threw this hypothesis into a still more definite form, namely, that the ions which diffused into muscular tissue entered into combination with some constituent of the tissue. The evidences upon which this hypothesis was based were, first, the physiological dependence of muscular tissue upon the nature of the ions diffusing into it—a dependence which had already been demonstrated for the heart by the experiments of Ringer, Howell and Cooke, and Locke,⁴ and to a certain degree also for striated muscle by the experiments of Biedermann⁵; and, secondly, the definite reactions which could be attributed to definite ions—reactions which could not be accounted for on a purely physical basis. In a later paper⁶ these compounds with ions received the name "ion-proteids" and what may be regarded as the central conception of the ion-proteid hypothesis was reached, namely, "that we can impart to a tissue new properties by changing the quality and the relative proportions of the ions in combination with the proteids. The characteristic qualities of every tissue are partly due to the fact that its ion-proteids contain certain ions in definite proportions. Any change in this proportion is accompanied by a change in the properties of the tissue."⁷ Although there is no evidence to show that only ion-proteids are formed in the tissues—indeed there are experiments which appear to indicate the existence of other ion-compounds with organic substances⁸—yet the hypothesis that ion-compounds, or, in-

¹ Loeb, *ibid.*, lxxi, p. 457, 1898; lxxv, p. 303, 1899.

² *Ibid.*, lxxv, p. 303, 1899.

³ *Festschrift für Professor Fick*, Braunschweig, 1899, p. 101.

⁴ Ringer, *Journ. of Physiol.*, iii, pp. 195 and 380, 1880-82; iv, pp. 29, 222, and 370, 1882-83; vi, p. 154, 1885. Howell and Cooke, *ibid.*, xiv, p. 198, 1893. Locke, *ibid.*, xviii, p. 332, 1895.

⁵ *Sitzungsber. d. Wiener Akad.*, lxxxii, part iii, 1880.

⁶ Loeb, *Amer. Journ. of Physiol.*, iii, p. 327, 1900.

⁷ Loeb, *ibid.*, p. 330.

⁸ Stiles and Beers, *ibid.*, xiv, p. 133, 1905.

deed, ion-proteids, may be formed in living tissues has now received the support of a vast number of experimental results—experiments on the action of electrolytes upon almost every variety of tissue, which have been carried out by numerous investigators during recent years. From the control which we can exercise over living tissues by means of electrolytes it appears probable that the ions in combination in the tissue can, *in general*, be displaced by other ions when the tissue is acted on by suitable electrolytes.

Early this year I brought forward a theory of the genesis of protoplasmic motion¹ by which I endeavored to account for the movements of protoplasmic bodies by changes in surface-energy, due to the charges on the ions liberated by the ion-proteids on katabolism or dissociation. In the paper referred to, I advanced the idea that the nature of the ion-proteid formed *in general* under the influence of an electrolyte is determined by the velocities of the ions into which the electrolyte is dissociated, since the swifter-moving ions would diffuse into the tissue in greater numbers than the slower-moving ions. This hypothesis really consists of two parts:

(a) That the ion-proteids² are compounds of a proteid-radical and an ion. The ion-proteid once formed, in subsequent reactions the ion-proteids formed are substitution-compounds in which one ion has been substituted for another.

(b) That the ion-proteids obey some form of the mass-law in their formation, inasmuch as the ion which enters the tissue in the greatest quantity is assumed to take the major share of the proteid.

Both parts of the hypothesis, but particularly part (b), were tested by and appear to receive support from my experiments on the influence of electrolytes on the frequency of the heart-beat.³

Supposing this hypothesis to be correct, then we should expect to find that the ion-proteid compounds have very definite chem-

¹ *Trans. of the Roy. Soc. of South Australia*, xxix, p. 1, 1905.

² Although I generally use the term ion-proteid, I wish it to be clearly understood that I do not limit myself to ion-proteid. On the contrary, I fully recognize the possibility of the existence of other ion-colloid compounds.

³ *Pflüger's Arch. f. d. ges., Physiol.*, cx, p. 610, 1905

ical characters stamped on them by the ion which has entered into combination. Just as phenol by the substitution of three of its hydrogen atoms by three nitro-groups, with the formation of picric acid, has its acid properties greatly increased, and as ammonia and phosphine have their basic properties greatly increased by the substitution of methyl or ethyl groups for one or more of their hydrogen atoms, so we should expect the ion-proteid compounds, composed as they are of a highly inert proteid moiety in combination with a chemically active moiety, that is, an ion, to exhibit more or less basic or acid properties according to the basicity or acidity of the *ion* in the combination.

To put the suggestion in a more concrete form, if, *ex hypothesi*, the conditions of ionic velocity, etc., are such that we should expect a predominance of anion-proteids in the tissue, then since the anions are, in general, acid radicals, the tissue should present acid characters in the sense that it should have the power conferred upon it of combining with bases; moreover this should be the case even if the anions are OH' ions, for OH' combines with H⁺, Na⁺, K⁺, etc., which are basic radicals. Similarly when the ion-proteids in excess should be kation-proteids we should expect the tissue to present basic characters, in the sense that it should have the power of combining with acids; and this should be the case even when the kations are H⁺ ions, since these combine with Cl⁻, NO₃⁻, etc., which are acid radicals.

The first method for investigating the correctness of this hypothesis which suggested itself to me was to ascertain the influence of electrolytes upon the toxicity of alkaloids, which have pronounced basic characters and also, in many cases, phenolic characters. A preliminary report of my experiments in this direction was published early in October,¹ and a full account of my results will be embodied in a paper which is to appear in a subsequent number of this Journal. I had not proceeded far, however, when it struck me that if the power of the ion-proteid to combine with acids or bases could be modified at will by treating the tissues with appropriate electrolytes, then it should be possible to reveal these changes by using a color-acid or color-base as an indicator, judging the basicity or acidity of the tissues by their staining-power. I at first used iodine-

¹ *University of California Publications, Physiology*, ii, No. 17, 1905.

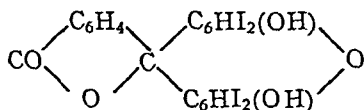
eosin, which is a weak color-acid, and a very delicate test for bases.¹ As iodine-eosin must be used in chloroform-solution, however, the technical difficulties were increased, while owing to its being an insufficiently weak acid the neutral point was not very clearly defined. I shall allude to this point more fully in the theoretical discussion which follows, but the difficulties encountered in the use of iodine-eosin led me to try methyl green. The methyl-green method, inasmuch as methyl green is a very weak base, gave much better results and the neutral point could be determined with considerable accuracy: the technique was also simplified since methyl green can be used in aqueous solution.

I fully recognize that I have as yet barely touched the experimental possibilities which appear to me to be opened out by the methyl-green method of determining the nature of the ion-proteids in a tissue. I hope to publish further investigations on the subject later; but it appeared to me that the publication of the results so far obtained would render the meaning of my experiments with alkaloids much clearer and place the whole argument on a firmer basis.

II. EXPERIMENTAL.

1. *Experiments with Iodine-Eosin.*

Iodine-eosin is the free color-acid, tetraiodo-fluorescein,



It is orange in color and insoluble in water but is soluble in chloroform or in ether. In contact with bases it forms the deep red salt erythrosin, which is insoluble in ether or chloroform.²

This substance was used in 1889 by Mylius³ for determining the alkalinity of glass. Ehrlich, in 1898, was the first to apply the method to histology, demonstrating that the cytoplasm of

¹ Vide Gustav Mann, *Physiological Histology*, Oxford, 1902, p. 214.

² Gustav Mann, *loc. cit.*, p. 430.

³ *Ber. d. deutsch. chem. Gesellsch.*, xxii, p. 310, 1889.

leucocytes and the blood-platelets stained deeply, while the red blood corpuscles and the nuclei of the white corpuscles remained colorless.¹ Later, Mann utilized it to demonstrate the basic nature of pure albumin crystals and also investigated the basicity of different tissues of a rat.² In 1900 Hoff investigated by this method the distribution of alkalies in plant tissues.³

The object of my experiments was to ascertain the influence of various electrolytes upon the power of infusoria, which had been subjected to their action, to stain in iodine-eosin. The following was the method employed.

About 0.5 c.c. of culture full of infusoria was placed in a test-tube and 12 c.c. of the solution, the influence of which was to be investigated, were added and the contents of the tube shaken. This was allowed to stand for 10 minutes and then centrifuged at a moderate rate. The infusoria were thus collected in a thick cluster at the bottom of the tube. The supernatant fluid was then poured off and 12 c.c. more of the solution under investigation were added: the tube was shaken and allowed to stand as before for 10 minutes, again centrifuged, and the process repeated a third time. After pouring off nearly all the supernatant fluid for the third time, a drop of the fluid, left at the bottom and very thickly inhabited by infusoria, was placed on a slide and dried at a temperature of 68°-70° C. The slide was then placed in distilled water for 5 minutes to wash off the salt or other electrolytes which had crystallized out in the drying, and was then dried again at 68°-70° C. The slide was then placed in a 0.33 per cent. solution of Grübler's iodine-eosin in chloroform (Kahlbaum's C. P.) and left for 5 minutes. It was then taken out and placed in pure chloroform for 20 minutes to wash out the free color-acid, then dried and mounted in xylol-balsam. The infusoria used were always taken from the top layer of the same culture which contained both paramœcia and colpoda in great abundance.

The xylol solution of Canada balsam used for mounting was carefully tested with iodine-eosin itself and found to be neutral. The degree of staining was always noted as soon as possible after mounting to avoid any changes due to fermentation processes taking place in the balsam on standing, although all the specimens prepared were afterwards compared in order to confirm the first judgment.

(a) *Experiments with Salts.*

The following is a table of the results which were obtained when the organisms were treated as described with solutions of salts.

¹ Gustav Mann, *loc. cit.*, p. 214.

² Gustav Mann, *loc. cit.*, p. 214.

³ *Botan. Centralbl.*, lxxxiii.

TABLE I.

Salt. ¹	Reaction of salt in normal concentration.	Paramœcium.	Colpodium.
$\frac{N}{5000}$ MnCl ₂	Very faintly acid to litmus	Cytoplasm deep red	Cytoplasm deep red
$\frac{N}{50}$ MgCl ₂	Neutral to litmus	" pink	—
$\frac{N}{50}$ MgSO ₄	Neutral to litmus and to phenolphthalein	" pink	" pink
$\frac{N}{5000}$ CuSO ₄	Acid to litmus	" deep red	" deep red
$\frac{N}{5000}$ ZnSO ₄	Acid to litmus	" pink	" pink
$\frac{N}{50}$ Na ₂ SO ₄	Neutral to litmus and to phenolphthalein	" pink	" pink
$\frac{N}{50}$ NaCl	Neutral to litmus and to phenolphthalein	" pink	" pink
$\frac{N}{50}$ NaF	Neutral to litmus and to phenolphthalein	" pink	" pink
$\frac{N}{50}$ KBr	Neutral to litmus and to phenolphthalein	" pink	" pink
$\frac{N}{50}$ KI	Neutral to litmus and to phenolphthalein	" pink	" pink
$\frac{N}{50}$ NH ₄ Cl	Faintly acid to litmus	"pink or red	" pink or red
$\frac{N}{50}$ NH ₄ NO ₃	Faintly acid to litmus	" pink	" pink
$\frac{N}{50}$ K ₂ SO ₄		" deep red	" deep red
$\frac{N}{50}$ KNO ₃	Neutral to litmus and to phenolphthalein	" deep red	" deep red
$\frac{N}{50}$ CH ₃ COOK	Alkaline to litmus and to phenolphthalein	" deep red	" deep red
$\frac{N}{50}$ (COONa) ₂	Neutral to litmus, faintly alkaline to phenolphthalein	" deep red	" deep red

It will be observed that the different salts exert a distinct influence upon the staining power—potassium sulphate, potassium nitrate, potassium acetate, sodium oxalate, manganous chloride, and copper sulphate being accentuators.²

If the washing of the slide in distilled water after the first drying was omitted quite different results were obtained, the reaction of the salt itself determining the color. Thus, if wash-

¹ The MnCl₂, MgSO₄, ZnSO₄, KBr, and NH₄Cl were Merck's C. P.; the MgCl₂ was de Haën's C. P.; the CuSO₄, Na₂SO₄, NaF, NH₄NO₃, K₂SO₄, KNO₃, CH₃COOK, and (COONa)₂ were Kahlbaum's C. P., while the NaCl and KI were Baker and Adamson's C.P.

² Vide Gustav Mann, *loc. cit.*, p. 212.

ing is omitted paramoecia which have been treated with magnesium chloride will not stain at all in iodine-eosin.

(b) *Experiments with Acids and Alkalies.*

TABLE II.

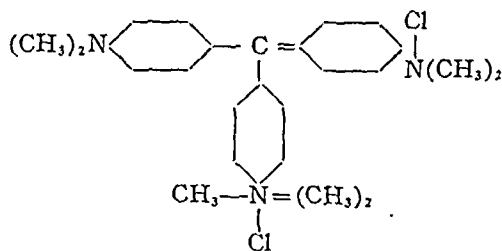
Electrolyte.	Paramoecium.	Colpodium.
$\frac{N}{50} C_2H_5COOH$	deep red	deep red
$\frac{N}{50} CH_3COOH$	deep red	deep red
$\frac{N}{5000} HCl$	deep red	deep red
$\frac{N}{10000} HCl$	deep red	deep red
$\frac{N}{5000} Ba(OH)_2$	deep red	deep red

These three acids as well as the barium hydrate were Kahlbaum's C. P. All appear to act as accentuators.

I asked a friend to place seven numbered but not labelled slides in the order of the depth of coloration, the most deeply colored being placed first. He placed them in the following order, the names being those of the salts with which the infusoria had been treated:—Sodium oxalate, potassium sulphate, potassium acetate, sodium chloride, ammonium chloride, sodium sulphate, magnesium chloride. In these and the following experiments only intact and uninjured individuals were compared, for it was observed that in toxic salts the disintegrated individuals were not always the same color as the uninjured individuals.

II. *Experiments with Methyl Green.*

Methyl green is the chlormethylate of hexamethyl-pararos-anilin chloride and its constitution is probably



Hence it has three amidogen groups, two of which contain a pentavalent nitrogen atom attached to the acid chlorine, while the third contains a trivalent nitrogen atom which gives a feeble basicity to the whole dye molecule. The dye thus stains acid tissue-elements such as nuclein compounds. Since the amidogen

groups of the methyl green molecule are not readily attacked chemically, it is not acted on by the normally very feebly acid cytoplasmic elements, but stains the comparatively strongly acid nuclei intensely.¹

The method employed in these experiments was as follows:

0.5 c.c. of culture was placed in a test-tube in 12 c.c. of the solution the action of which was being tested and, after shaking, was allowed to stand for 10 minutes and then centrifuged, etc., three times as in the experiments previously described. After the supernatant fluid had been poured off the third time, a drop of the concentrated infusorial suspension was placed on a slide and dried at 68°-70° C. The slide was washed in distilled water for 5 minutes, and then, after the superfluous moisture had been removed with filter-paper, it was placed in a 0.2 per cent. watery solution of Grübler's methyl green for 10 minutes, after which it was again washed in distilled water for 10 minutes. After removal of superfluous fluid by filter-paper, the preparation was mounted in glycerin and examined within the next 12 hours. As a check all the preparations were finally compared and found to have undergone no sensible alteration.

The following is a table of the results which were obtained when the organisms were treated as described with solutions of salts.

TABLE III.

Salt.	Paramœcium.	Colpodium.
$\frac{N}{\text{CaCl}_2}$	Cytoplasm fairly deep green though not so deeply stained as nucleus.	Cytoplasm less intensely green than paramœcium, nucleus deep green.
$\frac{N}{\text{MnCl}_2}$	Cytoplasm colorless or very faint green, nucleus deep green.	Cytoplasm colorless, nucleus deep green.
$\frac{N}{\text{MgCl}_2}$	Cytoplasm deep green, nuclei well stained.	Cytoplasm deep green, nuclei well stained.
$\frac{N}{\text{LiCl}}$	Cytoplasm faint green, nuclei well stained.	Cytoplasm faint green, nuclei well stained.
$\frac{N}{\text{MgSO}_4}$	Cytoplasm deep green, nuclei still more intensely stained.	Cytoplasm deep green, nuclei more intensely stained than cytoplasm.
$\frac{N}{\text{CuSO}_4}$	Cytoplasm very faint green, nucleus deep green.	Cytoplasm colorless, nucleus deep green.
$\frac{N}{\text{ZnSO}_4}$	Cytoplasm faint green, some individuals distinctly green; nucleus deep green.	Cytoplasm colorless, nucleus deep green.

¹ These facts are derived from Gustav Mann, *loc cit.*, pp. 424 and 459.

TABLE III (Continued)

Salt	Paramœcium	Colpodium
$\frac{N}{\tau\sigma}$ Na_2SO_4	Cytoplasm faint green, nucleus well stained.	Cytoplasm faint green, nucleus well stained.
$\frac{N}{\tau\sigma}$ NaCl	Cytoplasm faint green, nucleus well stained.	Cytoplasm faint green, nucleus well stained.
$\frac{N}{\tau\sigma}$ NH_4Cl	Cytoplasm faint green, nuclei not very distinct, some colorless.	Cytoplasm colorless, nucleus green.
$\frac{N}{\tau\sigma}$ KCl	Cytoplasm faint green, nucleus well stained.	Cytoplasm colorless, nuclei well stained.
$\frac{N}{\tau\sigma}$ K_2SO_4	Cytoplasm unstained or in some individuals barely perceptibly green. Nucleus much less stained than in CaCl_2 but still distinctly green.	Cytoplasm unstained, nucleus much less stained than in CaCl_2 but still distinctly green.
$\frac{N}{\tau\sigma}$ KNO_3	Cytoplasm unstained, nucleus stained.	Cytoplasm unstained, nucleus stained.
$\frac{N}{\tau\sigma}$ CH_3COONa	Cytoplasm unstained, frequently even nucleus barely perceptibly stained	Cytoplasm unstained, frequently even nucleus barely perceptibly stained.
$\frac{N}{\tau\sigma}$ CH_3COOK	Cytoplasm and nucleus of majority absolutely unstained—in a few individuals nucleus faint green.	Cytoplasm and nucleus absolutely unstained.
$\frac{N}{\tau\sigma}$ $\text{CH}_3\text{CH}_2\text{CH}_2\text{COONa}$	Cytoplasm and nucleus absolutely colorless.	Cytoplasm and nucleus absolutely colorless.
$\frac{N}{\tau\sigma}$ KH_2PO_4	Cytoplasm light green, nuclei well stained.	Cytoplasm faint green, nuclei well stained.
$\frac{N}{\tau\sigma}$ Na_2HPO_4	Cytoplasm and nucleus absolutely unstained except in 1 or 2 individuals in which nuclei faint green.	Cytoplasm and nucleus absolutely unstained except in 1 or 2 individuals in which nuclei faint green.
$\frac{N}{\tau\sigma}$ NaHCO_3	Cytoplasm and nuclei unstained save in 1 or 2 individuals in which nuclei very faint green.	Cytoplasm and nuclei unstained save in 1 or 2 individuals in which nuclei very faint green.
$\frac{N}{\tau\sigma}$ $(\text{NH}_4)_2\text{CO}_3$	Cytoplasm and nuclei unstained, only 1 or 2 stained slightly.	Cytoplasm and nuclei unstained, only 1 or 2 stained slightly.
$\frac{N}{\tau\sigma}$ $(\text{NH}_4)\text{CNS}$	Cytoplasm colorless or very faint green. In many, both nucleus and cytoplasm unstained; in others, nucleus slightly stained.	Cytoplasm and nucleus unstained.

The following is a table of the results with acids and alkalis.

TABLE IV.

Electrolyte.	Paramœcium.	Colpodium.
$\frac{N}{10}$ Glycollic acid	Cytoplasm faint green in most, in some colorless, nucleus deep green.	————
$\frac{N}{10}$ Propionic acid	Cytoplasm colorless, nucleus deep green, sometimes faint green granules in cytoplasm.	Cytoplasm colorless, nucleus deep green.
$\frac{N}{1000}$ NaOH	Cytoplasm faint green, in a few individuals darker green, nucleus deep green.	Cytoplasm faint green, nucleus deep green.
$\frac{N}{1000}$ Ba(OH) ₂	Cytoplasm faint green, in a few individuals darker green, nucleus deep green.	Cytoplasm faint green, nucleus deep green.

The solutions of electrolytes used in these experiments were the same as those used in the experiments with iodine-eosin, (p. 285); those which were not used in the experiments previously described were all made up from Kahlbaum's C. P. chemicals.

III. Experiments With Methyl Green on Gelatin Plates.

Plates of gelatin, about one millimeter thick and one centimeter square, were allowed to stand for three hours in finger-bowls containing fifty cubic centimeters each of the following solutions: $\frac{N}{2}$ potassium acetate, $\frac{N}{2}$ sodium acetate, $\frac{N}{2}$ potassium sulphate, $\frac{N}{2}$ potassium nitrate, $\frac{N}{2}$ ammonium chloride, $\frac{N}{2}$ magnesium sulphate, $\frac{N}{2}$ magnesium chloride, $\frac{N}{2}$ calcium chloride. They were then transferred to the same solutions containing four-tenths per cent. of Grüber's methyl green for half an hour each and then replaced in the salts alone for one hour and shaken occasionally to wash out excess of stain. The plates were then placed on glass over a white surface and the depth of color compared.

The gelatin which had been treated with potassium acetate was only slightly colored, and what color there was was of a more purple shade than the color of the others. The plate which had been treated with sodium acetate was the next most deeply stained, while that which had been treated with potassium sulphate followed. Between the depths of color of the rest it was difficult to distinguish. It will be seen that those which stained least were all plates which had been in solutions in which the kation has a higher velocity than the anion.

Before concluding these remarks, descriptive of the experimental results, I wish to point out that the power of infusoria to stain in iodine-eosin or in methyl green is in close parallelism with the transport-numbers for the anion of the electrolytes with which they have previously been treated. The electrolytes in Table I and in Table III down to the heavy line are arranged in the order of the transport-numbers for the anion, the electrolyte with the largest transport-number being placed first. After treatment with electrolytes in which the transport number is greater than 0.5 ($v > u$), the organism behaves in general as if its protoplasm were acid in character—staining deeply in the basic and but faintly in the acid dye. After treatment with electrolytes in which the transport-number is less than 0.5 ($u > v$), they behave in general as if the protoplasm were basic in character and stain deeply in the acid and but faintly or not at all in the basic dye. These facts will be considered more fully in the third or theoretical part of this paper.

III. THEORETICAL.

1. *The evidence that color-acids and color-bases may combine with some constituent of protoplasm.*

The idea that dyes combine with some constituents of the tissues they stain has prevailed since Miescher, in 1874, discovered that methyl green combines chemically with nucleic acid to form green insoluble color-salts.¹ This was followed by Ehrlich's discovery that only acid stains will color the α -granules of certain leucocytes, while only basic dyes will stain the γ -granules of the mast-cells, and only neutral dyes are taken up by the neutrophile or ϵ -granules.² Since then a great number of investigators have embraced the chemical theory of staining and have adduced a variety of facts in support of the theory.³

Fischer,⁴ on the other hand, has called in question the chem-

¹ Quoted after Gustav Mann, *loc. cit.*, p. 343.

² *Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes*, Berlin, 1891.

³ An historical account of the chemical and physical aspects of staining will be found in Gustav Mann, *loc. cit.*, pp. 330-343.

⁴ *Fixierung, Färbung, und Bau des Protoplasmas*, 1899; quoted after Gustav Mann, *loc. cit.*, p. 334.

ical view of staining and endeavored to explain all staining as a purely physical process of absorption. While it is quite possible that the compounds formed between the tissue-elements and dye-molecules may be of the nature of absorption-compounds (Van Bemmeln), yet Mann and others¹ have shown that it is impossible to explain many of the phenomena of staining upon a purely physical basis. Without attempting to enumerate the various arguments which have been advanced for and against the chemical theory of staining, which has already been done very fully by Mann in the work to which I have frequently referred, I may allude especially to the researches of Mathews.²

Mathews, experimenting upon albumoses and coagulated albumin in vitro, showed that color-acids possess the same albumin-precipitating powers as picric, molybdic, wolframic, or tannic acids, and the precipitate is colored in a manner characteristic of the stain. Since the sodium salts of the color-acids were generally used, the addition of a little acid (acetic acid) to liberate the free color-acid was necessary to bring about the reaction. In the same way, if salts of the acids referred to above are used, a little acid must be added before they throw albumin out of solution. Basic dyes, on the contrary, only combined with albumin in the presence of alkalies.

Similar considerations were found to hold good for tissues which had been hardened in alkaline, neutral, or acidulated alcohol, while Mathews attributed the staining of nucleins by basic dyes to the presence of unsaturated nucleic acid. The experiments of Lilienfeld upon the artificial nucleins are also in confirmation of this view; so long as the artificial nucleins are not saturated with albumin they stain in methyl green; after saturation with albumin they show a greater affinity for acid dyes.³

The chemical theory of staining, quite apart from the independent possibility of physical staining, therefore rests upon a firm basis of observation and experiment and Mann concludes "that, either accompanying the physical staining or quite

¹ Gustav Mann, *loc. cit.*, p. 336 and ff. and p. 352. Mathews, *Amer. Jour. of Physiol.*, i, p. 445, 1898.

² Mathews, *loc. cit.*

³ Lilienfeld, *Arch. f. Physiol.*, p. 391, 1893.

apart from it, true chemical staining is not only possible but actually does occur." ¹

2. *The evidence that electrolytes may influence the staining power of tissues.*

In discussing Mathews' results I have already indicated some of the evidence that electrolytes may influence the staining power of tissues. But the use of electrolytes as mordants has long been well known among professional dyers. For example, we have color-acids and acid salt-colors used as mordants for basic dyes, and the alums, sulphates of iron, acetates of iron, copper, aluminum, and chromium, chromium-compounds, and potassium-antimonyl tartrate as mordants for acid dyes. These mordants have also been more or less extensively used in the staining of tissues.

Still more important in this connection are the accentuators, substances which "by acting in some not always explainable manner on the material or the stain" produce or accentuate the affinity of the tissue for the dye.² For example, caustic potash used in staining tubercle bacilli with methylene blue by Koch's method; borax, also used as an accentuator for methylene blue; anilin, used by Ehrlich as an accentuator for gentian violet; soap, used by Nissl in staining the basophile granules in nerve-cells with methylene blue; pyridin and quinolin, also used as accentuators for basic dyes; acetic acid, used in staining with dahlia or fuchsin; phenol, used in staining with fuchsin; and oxalic acid, used in staining with methyl violet.³ It will be observed that, in general, the accentuators for basic dyes are basic substances or compounds of weak acids with strong bases (as, for example, borax), while the accentuators for acid dyes are acid substances. Strongly basic dyes, such as toluidin-blue, must be used in acid solutions if only nuclein-compounds are to be stained⁴; while Mathews has pointed out that albumoses, coagulated albumin, and the cytoplasm of cells stain intensely in basic stains such as methyl green, safranin, methyl blue, toluidin-blue, and thionin only when used in alkaline solution.⁵

¹ *Loc. cit.*, p. 366.

² Mann, *loc. cit.*, p. 212.

³ Vide Mann, *loc. cit.*, p. 212.

⁴ Mann, *loc. cit.*, p. 215.

⁵ Mathews, *loc. cit.*

Burchardt¹ found that if intestines were left for twenty-four hours in two to five per cent. acetic acid and then were washed in water and hardened in alcohol, basic dyes would no longer stain the nuclear chromatin. Similarly, while fixation by sublimate alone gave good staining with methyl green, fixation by sublimate + acetic acid did not.

Victor Henri and des Buncels² have investigated the influence of zinc nitrate and sulphate and of sodium nitrate and sulphate upon the depth of stain imparted to gelatin plates by anilin blue. They find that the plates stain deeply when they have been treated with zinc sulphate or nitrate and only feebly when they have been treated with sodium nitrate or sodium sulphate.

Recently Höber and Grünspun³ have published a preliminary communication in which they describe experiments conducted with a view to ascertaining the influence of electrolytes upon the chemical condition of nervous tissue in the frog. They immersed pieces of nerve in various saline solutions for twenty-four hours and then stained them in toluidin-blue. They find that the axis-cylinders stain most deeply when the nerves have been immersed in sodium salts (chlorides, acetates, sulphates, and tartrates), especially in sulphates and tartrates. The axis-cylinders stain much less deeply when they have been immersed in ammonium and potassium salts of the same acids, while lithium salts lie between sodium and ammonium salts in their effect upon the staining power.

The experiments upon infusoria which I have described and those of Höber and Grünspun show that an electrolyte may influence the staining power of a tissue apart from the acid or basic properties of the electrolyte in question. That the hydrogen or hydroxyl ions in the solution do not alone determine the staining power is shown, for instance, by the fact that infusoria which have been treated with potassium acetate do not stain at all in methyl green, and the same is true for those which have been treated with ammonium thiocyanate. Now potassium acetate is alkaline, while a solution of ammonium thiocyanate, judging from the relative strengths of thiocyanic acid and of

¹ *La Cellule*, xii, p. 335, 1897, quoted after Mann, *loc. cit.*, p. 351.

² *Compt. Rend. d. Soc. Biol.*, lxi, p. 132, 1905.

³ *Zentralbl. f. Physiol.*, xix, p. 390, 1905.

ammonium hydrate, should contain an excess of hydrogen ions. On the other hand, infusoria in solutions of calcium chloride, magnesium chloride, etc., which contain an excess of hydrogen ions over hydroxyl ions, stain deeply in methyl green. Moreover infusoria which have been treated with acids or alkalies stain deeply in iodine-eosin, while in magnesium chloride, calcium chloride, etc., they stain only faintly, and in potassium acetate they stain deeply; similarly infusoria which have been treated with acids or alkalies stain only faintly in methyl green but those which have been treated with magnesium chloride or calcium chloride stain deeply, while those which have been treated with sodium acetate or potassium acetate stain only faintly or not at all.

Neither can it be argued that the various electrolytes affect the staining power of cells only by influencing the permeability of the cell-wall. For, on this hypothesis we should suppose, since paramoecia which have been treated with magnesium chloride stain much less intensely in iodine-eosin than those which have been treated with potassium acetate, that the cell-wall had been rendered less permeable by the magnesium chloride: but in contradiction to this we find that paramoecia which have been treated with magnesium chloride stain deeply in methyl green, while those which have been treated with potassium acetate do not stain at all.

In endeavoring to explain these facts, therefore, it appears that we must look for some chemical alteration in the tissue which may be brought about not only by hydrogen and hydroxyl but by other ions.

3. *The ion-proteid hypothesis.*

I have already, in the introduction, briefly outlined the reasons in support of the ion-proteid hypothesis. To briefly recapitulate, they were, firstly, the marked physiological effects of solutions of electrolytes, effects which could not be obtained in solutions of non-electrolytes,¹ and secondly, the definite reactions called forth by definite ions or groups of ions. Doubt appeared to be cast upon the existence of these ion-compounds by the in-

¹ As for example, rhythmic muscular contraction; vide Loeb. *Festschrift für Professor Fick*, Braunschweig, 1899, p. 101.

vestigations of Bugarsky and Liebermann,¹ who found that, although the conductivity of acid or alkaline solutions was notably diminished by the addition of albumin, sodium chloride solutions did not show any diminution of conductivity, which they considered sufficient to indicate the formation of an ion-compound. The differences in conductivity which they did obtain, however, are in the sense which would be indicated by the ion-proteid hypothesis; the quantity which may be supposed to combine may well be so small as to fall within the limits of experimental error or within the magnitude of differences in conductivity found by Arrhenius² on adding organic substances to solutions of electrolytes. Moreover, the evidence obtained from the analysis of tissues does not agree with Bugarsky and Liebermann's conclusions, for the ash of tissues is greater than the salt content as determined by the conductivity or kryoscopic methods,³ although, of course, it is possible that ion-compounds other than ion-proteids may account for the difference. As regards the marked diminution of conductivity observed by Bugarsky and Liebermann in acid and alkaline solutions, it was pointed out by Heynsius in 1876 that acids and alkalies combined with albumin in varying proportions⁴; and Starke has found that alkali combines in two proportions to form alkali-albumin and alkali *albuminate* respectively, although intermediate stages may exist⁵; in the first stage the alkali is just sufficient to keep the albumin in solution; at a higher concentration the albuminate is formed. I should be inclined to attribute the diminution in conductivity in acid and alkaline solutions found by Bugarsky and Liebermann to the formation of the albuminate rather than to the formation of the ion-compound, the influence of which might well be too small to detect in this way.

The experiments which I have described and those of Höber and Grünspun to which I have referred certainly appear to lend support to the ion-proteid hypothesis, for they afford chemical

¹ *Pfluger's Arch. f. d. ges. Physiol.*, lxxii, p. 51, 1898.

² *Zeitschr. f. physikal. Chem.*, ix, p. 487, 1892.

³ Taylor, *University of California Publications, Pathology*, i, p. 71 1904.

⁴ *Pfluger's Arch. f. d. ges. Physiol.*, xi, p. 624, 1875; xii, p. 549, 1876.

⁵ *Zeitschr. f. Biol.*, xl, p. 419, 1900.

evidence of chemical changes in tissues brought about by neutral salts in dilute solution as well as by acids and alkalies.

4. *The possible acid or basic character of tissues treated with solutions of electrolytes and its probable influence upon the staining-power.*

Assuming the truth of the ion-proteid hypothesis, as I have pointed out in the introduction, we might expect the acid or basic nature of the ion in combination to markedly affect the acid or basic properties of the ion-proteid molecule. If the ion in combination is a metal ion or hydrogen we should expect the resulting ion-proteid to be basic in its properties and to have an added affinity for acid radicals; while if the ion in combination be an acid radical or hydroxyl we should expect the resulting ion-proteid to be acid in its properties and to have an added affinity for basic radicals.

Assuming, also, the truth of the hypothesis that the ion present in the tissue in the greatest amount forms the greatest part of the ion-proteid, then, when the tissue is acted upon by a solution of an electrolyte in which the kation (metal or hydrogen) has a higher migration velocity than the anion (acid radical or hydroxyl), we might expect the major part of the ion-proteids formed to be basic in character; while in solutions in which the anion has a higher migration-velocity than the kation we might expect the major part of the ion-proteids formed to be acid in character.

Thus, if our hypotheses be correct, the relative velocities of the anion and kation in a solution of an electrolyte should exert a marked influence upon the power of the tissue to stain in color-acids or in color-bases. Hittorf's transport-number for the anion¹ is the ratio of the velocity of the anion to the sum of the velocities of the anion and kation ($\frac{v}{u+v}$), and if $\frac{u}{u+v}$ be the corresponding number for the kation, the numbers $\frac{v}{u+v}$ and $\frac{u}{u+v}$ are proportional respectively to the masses of anion and of kation which diffuse across a given area in a given time, and hence may be supposed to be proportional also to the masses respectively of anion and of kation which enter a tissue immersed in a solution of the electrolyte.

The following is a table of transport-numbers for the anion

¹ Vide Whetham, *Theory of Solution*, 1902, p. 208.

of most of the electrolytes which were used in the experiments described. The numbers obtained by different observers by different methods have not, according to the tables to which I have referred,¹ exactly the same absolute values, and I have therefore taken all the figures tabulated below from Hittorf's results for dilute solutions quoted by Fitzpatrick.² In every case the number for the least concentration quoted is taken—the concentration varying between about 0.01 and 0.1 equivalent gram-molecules per liter. The numbers for lithium chloride and sodium hydrate, however, are those determined by Kuschel and quoted by Fitzpatrick.

TABLE V.

Electrolyte.	Transport- No.	Electrolyte.	Transport- No.	Electrolyte.	Transport- No.
NaOH	.843	ZnSO ₄	.636	K ₂ SO ₄	.498
CaCl ₂	.683	Na ₂ SO ₄	.634	KNO ₃	.497
MnCl ₂	.682	NaCl	.622	CH ₃ COONa	.443
MgCl ₂	.678	KBr	.514	CH ₃ COOK	.324
LiCl	.674	KI	.511	HCl	.210
MgSO ₄	.656	NH ₄ Cl	.508		
CuSO ₄	.638	KCl	.503		

When the transport-number for the anion is greater than 0.5 of course the velocity of the anion is greater than that of the kation; when the number is less than 0.5, the velocity of the kation is greater than that of the anion.

I am unable to find any determinations of the transport-numbers for the other substances which I have used in these experiments, but according to Kohlrausch's latest figures³ for the ionic velocities, the velocity of CH₃COO' is less than that of Cl', while that of C₂H₅COO' is still less. Hence the transport-numbers of hydrochloric, acetic, and propionic acids probably decrease in the order named, while, according to the general

¹ Landolt-Börnstein, *Physikalisch-chemische Tabellen*, 1905. Fitzpatrick, *Table of Electro-chemical Properties of Aqueous Solutions*. Reprinted in Whetham's *Theory of Solution*, 1902, from *British Assoc. Report*, 1893; also Whetham, *loc. cit.*, p 212.

² *Loc. cit.*

³ Landolt-Börnstein, *loc. cit.*

rule that heavy organic ions move with a lower velocity the greater their weight, we may assume that sodium butyrate has a lower transport-number for the anion than either sodium acetate or sodium propionate. According to Kohlrausch's figures, also, the transport-number for the anion in ammonium thiocyanate should lie between the numbers given by Hittorf for potassium nitrate and sodium acetate. It will be observed that the salts in Table I and in Table III down to the heavy line are arranged in the order of their transport-numbers, and that, with the exceptions of manganous chloride and copper sulphate, paramoecia and colpodia, treated with iodine-eosin, stain pink when they have been subjected to the action of salts the transport-numbers of which are *greater* than 0.5, and stain deep red when they have been subjected to the action of salts the transport-numbers of which are *less* than 0.5. Conversely, with the exceptions of manganous chloride, lithium chloride, and possibly copper sulphate and zinc sulphate, the depth of staining of these organisms in methyl green runs parallel with the magnitude of the transport-numbers of the salts with which they have been treated, varying from deep green staining of both cytoplasm and nucleus when they have been treated with calcium chloride, magnesium chloride, or magnesium sulphate through intermediate stages in which the nucleus only is stained or the cytoplasm is only faintly stained, to the total absence of staining-power in either cytoplasm or nucleus when they have been treated with potassium acetate or with sodium butyrate.

Recalling that according to my hypothesis the ion-proteid should be basic in character when the migration-velocity of the kation is higher than that of the anion, that is, when the transport-number is less than 0.5, and that it should be acid in character when the transport-number is greater than 0.5, the facts are certainly in very fair accordance with the hypothesis.

As I have pointed out, the salts of heavy metals, the action of which I have investigated, appear to be exceptions to the rule. Although the anions have higher velocities than the kations in these instances, yet the cells which have been treated with them would appear to be strongly basic judging from the intensity with which they stain in iodine-eosin and the fact that they stain only slightly in methyl green. Possibly in analogy with a

number of well-known inorganic reactions the ion-compounds of proteid with heavy metals are insoluble and, being removed from the sphere of chemical action, the reaction between proteid and heavy metal proceeds nearly to a completion, when practically all the proteid has been precipitated, so that here no equilibrium is reached and the ion present in the greater mass does not take the greater share of the proteid. Why lithium chloride should also be an exception (see Table III) I am at a loss to understand. Lithium chloride, however, occupies an exceptional position among salts in many other respects.

Acids and alkalies, so far as I have investigated, would also appear to be exceptions—barium hydrate being an exception for iodine-eosin (see Table II) and glycollic acid and to a less degree propionic acid being exceptions for methyl green (see Table IV). Possibly this is due to the formation of albuminates to which I have referred. In case it might be due to the formation of salts with traces of free acid or base not washed out of the tissue, I have tried washing the infusoria treated with acid or alkali and stained in iodine-eosin with absolute alcohol after the excess of free color-acid had been washed out in chloroform. Since the salts of iodine-eosin are soluble in alcohol¹ we would expect that the color-acid combined with other bases than proteid should be washed out in this way. I have, however, failed to detect any difference after washing twenty minutes in alcohol between paramœcia stained in iodine-eosin after treatment with hydrochloric acid and paramœcia which had been similarly stained after treatment with barium hydrate; in fact the depth of color of the cells was little, if at all, diminished by this treatment. I may point out that the fact that cells which have been treated with acids stain deep red in iodine-eosin shows that the iodine-eosin color radical has not merely combined with the excess of *free* hydrogen ions in the tissue, for the result would simply be the formation of the free color-acid, which is orange in color, not red.

5. *Possible objections to the method used; precautions to be taken.*

One of the most important objections which might be urged against the method which I have used is that the proteids are coagulated before staining and that the reaction of solutions of

¹ Mann, *loc. cit.*, p. 430.

proteids changes on heating to coagulation.¹ It does not appear to me certain, however, that this reaction-change in the solution may not be due merely to the removal of the proteid from a condition in which it can affect indicators added to the solution to a condition in which it cannot. As a test, however, I dried infusoria which had been treated in the usual way with sodium acetate, on a slide at room temperature, and then heated to 110° C. according to Ehrlich's method.² There is considerable difficulty in making the infusoria stick to the slide when treated in this way, especially as the operation has to be repeated twice in order to wash out the salt. These infusoria, however, stained just as faintly in methyl green as those which had been fixed by heating in the moist state. The basicity of infusoria after treatment with potassium acetate is, therefore, not an artifact due to heating. The experiments with gelatin are also not open to this objection.

The precautions which must be used if this method is to give reliable information as to the distribution of ion-proteids in a tissue are as follows:

(1) If a method of fixation which it is suspected may involve a reaction-change is used, controls should be made in which some fixation-method is used in which no reaction-change takes place.³ Preferably, fresh and unfixed tissues should be used, but this is not generally possible for reasons which will be found under (4).

(2) There should always be a large volume of the electrolyte, the influence of which we wish to determine, as compared to the volume of the tissue—so that the salts in the tissue can be neglected.

(3) Ample time should be allowed for the ions of the electrolyte to penetrate and to displace those already in combination.

(4) The excess of salt must all be removed from the tissue before applying the stain, as otherwise the reaction of the salt itself will determine the staining. Since this cannot always readily be done, when the proteids are still exchanging ions,

¹ Cohnheim, *Chemie der Eiweisskörper*, 1900, p. 5.

² Gustav Mann, *loc. cit.*, p. 143.

³ For methods, vide Gustav Mann, *loc. cit.*, p. 142.

without altering the proportions of the ion-proteids, it is, in general, necessary to use some fixation-method.

(5) The permanency of the stain when the tissue is exposed to the action of different solvents should be investigated in order to make sure that the staining is not merely due to the formation of a salt with free base or acid (not proteid) in the tissue. This would also serve to distinguish between purely physical staining and staining that really depends upon the chemical nature of the ion-proteids.

(6) The hydrolytic dissociation of the salt used must be taken into account, as the rapidly moving H^+ or OH^- ions will influence the proportion of anions to kations entering the tissue. General precautions such as the use of perfectly neutral solvents for stains, fixatives, and mounting media I need hardly do more than mention.

6. Chemical and physiological significance of the results. Some possible applications.

We have seen that in general the staining-power of a tissue in iodine-eosin or in methyl green runs parallel in a definite sense with the transport-number for the electrolyte. In terms of the hypothesis which I have put forward the mass of anion-proteid, or proteid of acid character, which is formed is proportioned to the mass of anions acting upon it, that is to $\frac{v}{u+v}$, while the mass of kation or basic ion-proteid is proportional to $\frac{u}{u+v}$; hence if A = mass of acid ion-proteid, and B = mass of basic ion-proteid, $A - B = \frac{v-u}{u+v} = \frac{2v}{u+v} - 1 = 2 \left(\frac{v}{u+v} - 0.5 \right)$. The results are, in general, those which would be indicated by the hypothesis. Infusoria which have been treated by electrolytes in which the anion has a higher velocity than the kation, that is $\frac{v}{u+v} > 0.5$, stain as if they were acid in reaction, while those which have been treated by electrolytes in which the kation has a higher velocity than the anion, so that $\frac{v}{u+v} < 0.5$, stain as if they were basic. Moreover there is a gradation in the staining-power, corresponding closely to the order of the transport-numbers.

These experiments therefore certainly appear to support the hypothesis of the existence of unstable ion-compounds in protoplasmic bodies. It seems very probable, also, that the ion in the compound exerts a definite influence upon the "pseudo-

acid" or "pseudo-basic" properties of the molecule. Mann has arrived at a very similar conclusion; he suggests that in the case of acetic acid the hydrions and acetanions "acting on the colloidal, chemically inactive, pseudo-acid, pseudo-basic proteid convert it into an electrolyte. Proteid, changed in this manner, can readily interact with the ions derived from the salt which we employ as a dye, and in consequence chemical union between the kations of the tissue and the anions of the dye (or the anions of the tissue and the kations of the dye) can readily take place."¹ The fact that the mere immersion of a living tissue in a solution of an electrolyte changes its chemical characters to such a marked degree shows that the ions in the ion-proteid molecules are readily replaced by other ions. The fact that we can predict in most cases the sense in which the pseudo-acid or pseudo-basic properties will be altered from the transport-numbers for the electrolyte renders it very probable that these ion-compounds obey some form of the mass-law,—the ion present in the tissue in the greatest mass forms the greatest part of the ion-proteids.

Staining methods have for some time been used, especially by Ehrlich,² for detecting acid or basic elements in tissues. The marked influence which electrolytes exert upon staining-power would appear to indicate that these acid or basic elements are the ion-compounds which we have been considering. It appears possible therefore, that staining-methods may, with the precautions which I have outlined, be utilized with toxicity experiments to ascertain the distribution of ion-proteids in various tissues. The abundance of basophile granules in nerve-cells may indicate the presence of anion-proteids. The fact that spongioplasmic structures stain especially deeply in hæmatoxylin³ may indicate the presence of excess of basic or kation-proteids, the presence of which is also revealed by the demarcation current and the "wave of negativity" in nerves and in muscle.⁴ Not only may this method be used to reveal the nature of the

¹ Gustav Mann, *loc. cit.*, p. 337.

² Ehrlich, *loc. cit.*

³ Schäfer, *Essentials of Histology*, 1902, p. 105.

⁴ Loeb, "The Physiological Problems of To-day." Address before American Society of Naturalists, Ithaca, 1897. Reprinted in *Studies in General Physiology*, ii, p. 497, 1905.

Robertson, *Trans. Roy. Soc. South Australia*, xxix, p. 29, 1905.

ion-proteids in normal tissues, but in tissues which have been placed under determinate conditions as in the experiments herein described.

IV. CONCLUSIONS.

1. The influence of various salts and of acids and alkalies upon the staining-power of infusoria and of gelatin plates in iodine-eosin and in methyl green has been investigated.

2. The various electrolytes exert a distinct and definite action upon the staining-power in the above-mentioned dyes. In general, after treatment with electrolytes in which the transport-number for the anion is *greater than* 0.5, the infusoria stain deeply in methyl green and faintly in iodine-eosin. After treatment with electrolytes in which the transport-number for the anion is *less than* 0.5 the infusoria stain deeply in iodine-eosin and faintly in methyl green. There is a general parallelism between the staining-power and the transport-number of the electrolyte with which the tissue has been treated.

3. The effects observed are not due, at least in their entirety, to alterations of permeability or to the hydrions and hydroxylions produced by the hydrolytic dissociation of the salts.

4. The results are such as to lend support to the hypothesis of an unstable ion-compound in protoplasm, the ion of which is readily replaced by other ions, the ion present in the greatest mass forming the greater part of the compound, and the acid or basic properties of the compound being determined by the acid or basic properties of the ion.

5. The salts of heavy metals which were investigated, as well as alkalies in the case of iodine-eosin and acids in the case of methyl green, were exceptions to the rule of parallelism between staining-power and transport-numbers which was found in the case of infusoria which had been treated with other electrolytes. Possibly the heavy metals are exceptions because the reaction between the heavy metal ion and the proteid is an unbalanced one, the ion-proteid being precipitated as soon as it is formed, so that a small amount of dicuprion, for example, can precipitate the whole of the available proteid although sulphanions may be in excess. That acids and alkalies are ex-

ceptions may possibly be attributed to the formation of albuminates to which I have alluded.

6. Possible applications to physiological investigations have been pointed out.

Finally I wish to express my indebtedness to Dr. Loeb for the facilities and encouragement which he has extended to me in carrying out these experiments.

I.—RESEARCHES ON PYRIMIDINS: SOME 5-IODOPYRIMIDIN DERIVATIVES; 5-IODOCYTOSIN.

PLATE I.

(Thirteenth Paper.)

By TREAT B. JOHNSON AND CARL O. JOHNS.

(Contributions from the Sheffield Laboratory of Yale University.)

(Received for publication, December 19, 1905.)

The object of the work described in this paper was to prepare some 5-iodopyrimidin derivatives and investigate their behavior towards ammonia and organic bases. It was considered desirable to compare the reactivity of such iodine derivatives with that of the analogous bromine compounds. In the course of our work on pyrimidins, it was necessary to decide whether 5-iodopyrimidins might serve for syntheses in which the bromine derivatives could not be used.

Several iodine-substituted pyrimidins have been described in the literature,¹ but so far as the writer is aware no attempts have hitherto been made to prepare iodine derivatives by direct iodation. We have selected for our investigation three pyrimidin derivatives, in which the 4- and 5-positions are occupied by hydrogen, viz.: 2-ethylmercapto-6-oxypyrimidin,² (I); 2,6-dioxypyrimidin³ (Uracil, II.); and 2-oxy-6-amino-pyrimidin⁴ (Cytosin, III). Wheeler and Bristol have shown that when such pyrimidins⁵ are attacked by nitric acid or bromine it is the hydrogen atom occupying the 5-position that is substituted. It might be expected that they would be attacked by iodine in a similar manner to form the corresponding 5-iodopyrimidins.

We now find that the three pyrimidins selected can be con-

¹ Gabriel and Colman, *Ber. d. deutsch. chem. Gesellsch.*, xxxii, p. 1525, 1899; *ibid.*, xxxii, p. 2921, 1899; Emery, *ibid.*, xxxiv, p. 4178, 1901; Büttner, *ibid.*, xxxvi, p. 2227, 1903.

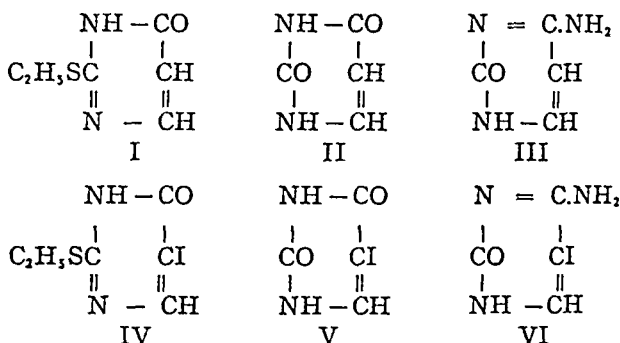
² Wheeler and Merriam, *Amer. Chem. Jour.*, xxix, p. 484, 1903.

³ Wheeler and Merriam, *loc. cit.*

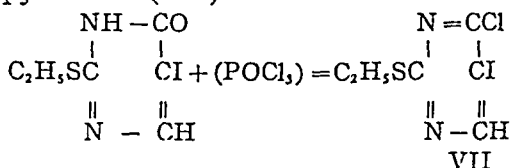
⁴ Wheeler and Johnson, *ibid.*, xxix, p. 492, 1903.

⁵ *Ibid.*, xxxiii, p. 437, 1905.

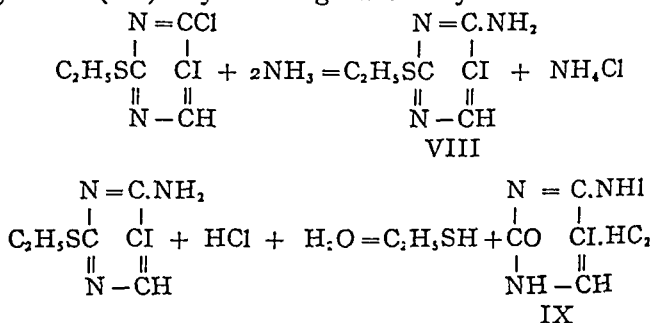
verted smoothly into 5-iodopyrimidins if treated with iodine in an alkaline solution.



In order to show that the iodine enters the pyrimidin ring in the same position (5) we have furthermore prepared 5-iodocytosin (VI) from 2-ethylmercapto-5-iodo-6-oxypyrimidin (IV). When this compound was warmed with phosphorus oxychloride it was converted quantitatively into 2-ethylmercapto-5-iodo-6-chlorpyrimidin (VII).



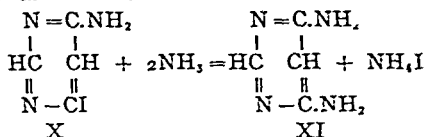
When this chlorpyrimidin was heated with alcoholic ammonia it reacted quantitatively to form 2-ethylmercapto-5-iodo-6-aminopyrimidin (VIII). This was then converted into 5-iodocytosin (IX) by boiling with hydrochloric acid. This



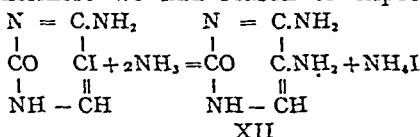
synthesis supports the assumption that the iodine in these compounds occupies the 5- and not the 4-position. Büttner¹ has shown that when an iodine occupies the 4-position in a

¹ *Loc. cit.*

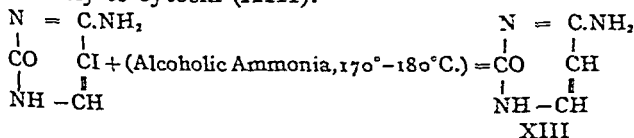
pyrimidin, it behaves as an imide-chloride, and can be replaced by an amino-radical. He prepared 4, 6-diaminopyrimidin (XI) by heating the corresponding 4-iodo-6-aminopyrimidin (X) with alcoholic ammonia.



It was of especial interest to investigate the behavior of 5-iodocytosin (VI) towards ammoniacal solutions, since it offered a possibility of preparing 2-oxy-5, 6-diamino-pyrimidin (XII). Furthermore we had reason to expect that 5-iodo-



cytosin (VI) would not show the same behavior towards aqueous and alcoholic ammonia. Johnson and Johns¹ have shown that such ammoniacal solutions show a remarkable difference in their reactivity. We now find that this iodo-pyrimidin (VI) can be heated with concentrated aqueous ammonia and be recovered unaltered. This result is in accord with an observation made by Wheeler and Johnson² in their examination of the properties of the corresponding 5-bromcytosin. On the other hand when 5-iodocytosin was heated with strong alcoholic ammonia under the same conditions with respect to temperature and time of heating, it was reduced practically quantitatively to cytosin (XIII).

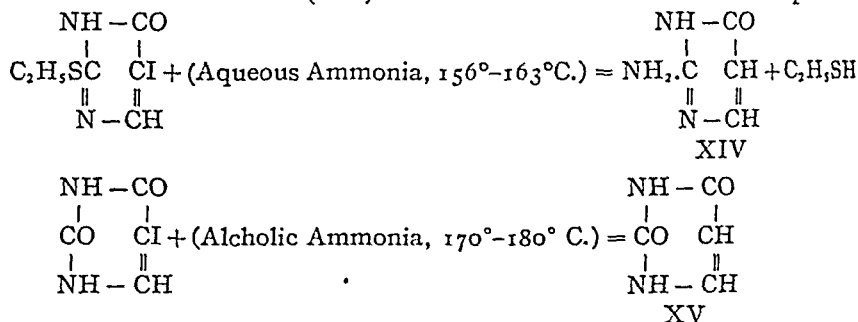


We now find that 2-ethylmercapto-5-iodo-6-oxypyrimidin (IV) and 5-iodouracil (V) likewise show a remarkable difference in behavior towards aqueous and alcoholic ammonia solutions. The mercaptopyrimidin (IV) can be heated with alcoholic am-

¹ *Ibid.*, xxxiv, p. 175, 1905.

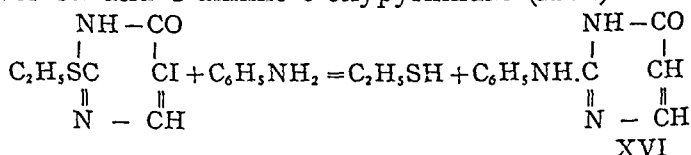
² *Ibid.*, xxxi, p. 591, 1904.

monia and recovered unaltered. On the other hand when heated with concentrated aqueous ammonia at the same temperature mercaptan was evolved and it was converted into 2-amino-6-oxypyrimidin (Isocytosin, XIV). 5-Iodouracil reacted in a reverse manner. When heated with aqueous ammonia it was recovered unaltered. On the other hand it was converted by alcoholic ammonia into uracil (XV). The action of alcoholic or aqueous



ammonia on 2-ethylmercapto-5-brom-6-oxypyrimidin¹ at temperatures between 140° and 200° C. does not give smooth results. Merriam² has shown that 5-bromuracil is converted smoothly into 5-amino-uracil when heated with aqueous ammonia.

We have observed that this replacement of iodine by a hydrogen atom can take place in the presence of aniline as well as ammoniacal solutions. When 2-ethylmercapto-5-iodo-6-oxypyrimidin (IV) was heated with aniline at 100° C., mercaptan was evolved and 2-anilino-6-oxypyrimidin (XVI) was formed.

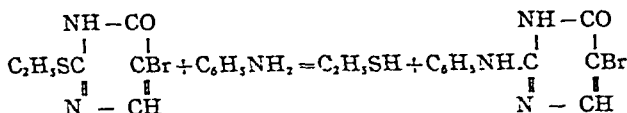


The same anilino-pyrimidin was obtained when 2-ethylmercapto-6-oxypyrimidin (I) was heated with aniline at 100° C. This abnormal result again illustrates the difference in behavior between 5-iodopyrimidins and their bromine analogues. The corresponding 2-ethylmercapto-5-brom-6-oxypyrimidin reacts quantitatively with aniline at 100° C. to form 2-anilino-5-brom-6-oxypyrimidin,³

¹ Wheeler and Johnson, *loc. cit.*

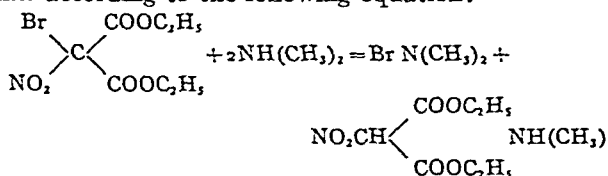
² *Amer. Chem. Jour.*, xxxi, p. 603, 1904.

³ Wheeler and Bristol, *ibid.*, xxxiii, p. 444, 1905.



It is a remarkable fact that the reduction of 2-ethylmercapto-5-iodo-6-oxypyrimidin to 2-ethylmercapto-6-oxypyrimidin should take place at a much lower temperature with aniline than with ammonia. In our experiments with iodocytosin we did not observe the formation of cytosin when it was heated with aniline. It was recovered unaltered after heating with aniline from 190° to 200° C.

We are not inclined to consider these abnormal replacements of iodine by hydrogen as exceptional cases of reduction. It seems probable to the writer that the mechanism of these interesting reactions is analogous to that when diethylbromnitromalonate is treated with amines. This ester was investigated by Willstätter and Hottenroth¹ in their attempts to synthesize Drechsel's diamino-acetic acid.² They observed that the bromine atom in this ester was extremely loosely bound, and it was reduced to diethylnitromalonate when treated with ammonia or fatty amines. They showed that it reacted with dimethylamine according to the following equation:



The ease with which the iodine in 2-ethylmercapto-5-iodo-6-oxypyrimidin (IV) could be replaced by hydrogen in the presence of amines suggested that this iodopyrimidin, under certain conditions, might be used for syntheses in reactions involving double decomposition. We now find that under the conditions employed in our experiments the iodine was very firmly bound and could not be replaced. For example: It could be heated with the potassium salt of phthalimid in alcohol, or with the sodium salt of urethane in benzene at 160° C. and be recovered unaltered. In these experiments it shows the stability of the corresponding

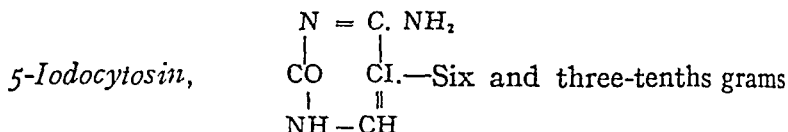
¹ Ber. d. deutsch. chem. Gesellsch., xxxvii, p. 1775, 1904.

² Beilstein, Handbuch, i, p. 1194.

For $C_4H_5O_2N_2I$ —

Calculated: N = 11.86 per cent.

Found: N = 11.77 per cent.



of synthetical cytosin¹ were dissolved in about 50 c.c. of water containing 3.3 grams of potassium hydroxide. To this solution were slowly added, with frequent shaking, 14.5 grams of finely pulverized iodine. The reaction was smooth and the iodocytosin began to separate immediately. After all the iodine had been added the solution was warmed on the steambath for a few minutes to complete the reaction. After neutralizing the free alkali with acetic acid the base was filtered off and recrystallized from boiling water. It separated on cooling in characteristic, branched crystals (Figure 1), which decomposed between 225° and 245° C. without effervescence, giving off iodine vapors.

It was practically insoluble in alcohol and benzene. The yield was quantitative. Analysis (Kjeldahl):

0.1188 gm. of substance gave 0.0210 gm. of nitrogen = 15 c.c. of $\frac{N}{16}$ HCl.

For $C_4H_4ON_2I$ —

Calculated: N = 17.72 per cent.

Found: N = 17.67 per cent.

Solubility of iodocytosin in water: One liter of water at 25° C. dissolved (1) 0.936 gram, (2) 0.988 gram.

Iodocytosin is very stable in the presence of boiling hydrochloric acid. One gram of the base was boiled with concentrated hydrochloric acid for two hours. The acid solution was then evaporated to dryness and the residue treated with a little dilute ammonia. We obtained the unaltered base which decomposed at from 220° to 235° C. Analysis (Kjeldahl):

0.1210 gm. of substance gave 0.02142 gm. of nitrogen = 15.3 c.c. $\frac{N}{16}$ HCl.

For $C_4H_4ON_2I$ —

Calculated: N = 17.72 per cent.

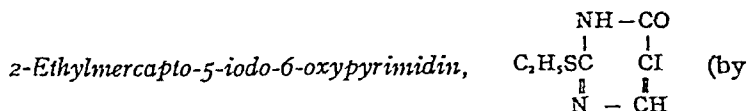
Found: N = 17.70 per cent.

Picrate of iodocytosin: The picrate crystallized from hot water in long needles. It had no definite melting point but

¹ Wheeler and Johnson, *loc. cit.*

bromine derivative—2-ethylmercapto-5-brom-6-oxypyrimidin. This compound and also 2-ethylmercapto-5-brom-6-amino-pyrimidin¹ could be heated with the potassium salt of phthalimid at 200° C. without any evidence of the formation of potassium bromide.

EXPERIMENTAL PART.

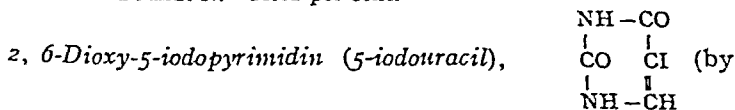


Dr. J. G. Statiropulos).—Ten grams of 2-ethylmercapto-6-oxypyrimidin,² were dissolved in 50 c.c. of water containing 3 grams of sodium hydroxide. To this solution was then slowly added 17 grams of pulverized iodine. After all the iodine had been added the solution was warmed on the steambath until the free iodine dissolved. The iodopyrimidin separated from the hot solution. After acidifying with acetic acid the solution was filtered and the pyrimidin purified by crystallization from alcohol. It deposited in slender prisms, arranged in crosses, and melted at 196° C. to a clear oil. It was insoluble in water, and readily soluble in benzene. The yield was practically quantitative. Analysis:

For $\text{C}_6\text{H}_7\text{ON}_2\text{SI}$ —

Calculated: N = 9.93 per cent.

Found: N = 10.02 per cent.



Dr. J. G. Statiropulos).—This compound was easily obtained when uracil was dissolved in alkali and treated with iodine as in the preparation of the above 2-ethylmercapto-5-iodo-6-oxypyrimidin. It crystallized from hot water in glistening scales, and decomposed at 272° C. It was soluble in hot alcohol. Analysis:

¹ Wheeler and Johnson, *loc. cit.*

² Wheeler and Merriam, *loc. cit.*

decomposed with effervescence at from 247° to 257° C. according to the rate of heating. Analysis (Kjeldahl):

0.1705 gm. of substance gave 0.03066 gm. of nitrogen = 21.9 c.c. $\frac{N}{10}$ HCl

For $C_4H_4ON_3I.C_6H_5O.N_3$ —

Calculated: N = 18.02 per cent.

Found: N = 17.98 per cent.

Acetic acid salt of iodocytosin: This salt is of special interest on account of its dissociation in a hot solution of acetic acid. Some iodocytosin was dissolved in hot acetic acid and the solution divided into two equal parts. The first part was then cooled quickly, when the unaltered base immediately separated in the form of irregular prisms. Analysis (Kjeldahl):

0.1253 gm. of substance gave 0.02198 gm. of nitrogen = 15.7 c.c. $\frac{N}{10}$ HCl.

For $C_4H_4ON_3I$ —

Calculated: N = 17.72 per cent.

Found: N = 17.54 per cent.

The second part was allowed to stand for several hours. The unaltered base separated at first in distorted prisms. On standing these prisms slowly assumed a new form and were transformed into large well developed prisms with distinct faces (Figure 2). They became opaque at about 110° C., and decomposed at from 220° to 240° C. according to the rate of heating. A nitrogen determination agreed with the calculated percentage in the acetic acid salt (Kjeldahl):

0.1320 gm. of substance gave 0.0189 gm. of nitrogen = 13.5 c.c. $\frac{N}{10}$ HCl.

0.1029 gm. of substance gave 0.01456 gm. of nitrogen = 10.4 c.c. $\frac{N}{10}$ HCl.

For $C_6H_5O_3N_3I$ —

Calculated: N = 14.14 per cent.

Found: N = (1) 14.31; (2) 14.15 per cent.

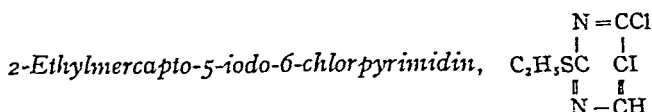
In order to decide whether the salt would dissociate again, it was dissolved in hot acetic acid and the solution cooled. The base was again obtained in the form of irregular prisms. Analysis (Kjeldahl):

0.0811 gm. of substance gave 0.01428 gm. of nitrogen = 10.2 c.c. $\frac{N}{10}$ HCl.

For $C_4H_4ON_3I$ —

Calculated: N = 17.72 per cent

Found: N = 17.60 per cent.



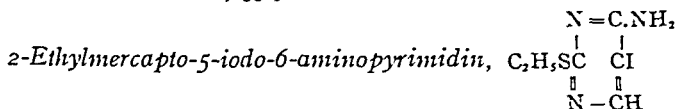
This compound was prepared by warming on the steambath 20 grams of 2-ethylmercapto-5-iodo-6-oxypyrimidin with 50 c.c. of phosphorus oxychloride. After the evolution of hydrochloric acid ceased the excess of phosphorus oxychloride was removed by heating at 100° C. under a pressure of 50 millimeters of mercury. We obtained a thick, brown varnish which immediately solidified when triturated with ice water. This contained iodine and melted at 68° C. to a turbid oil. It was purified by crystallizing from petroleum ether. It deposited in large prisms and melted at 69° C. to a clear oil. The yield was 20 grams, or 95 per cent. of the theoretical. Analysis (Kjeldahl):

0.2050 gm. of substance gave 0.01918 gm. of nitrogen = 13.7 c.c. $\frac{N}{16}$ HCl.

For $\text{C}_6\text{H}_5\text{N}_2\text{SClI}$ —

Calculated: N = 9.32 per cent.

Found: N = 9.35 per cent.



This compound was obtained when 10 grams of 2-ethylmercapto-5-iodo-6-chlorpyrimidin were heated with alcoholic ammonia for three hours at 128°–130° C. When the tube was examined the base had crystallized from the alcoholic solution in long, slender prisms. The crude material melted at 126° C. When allowed to crystallize slowly from alcohol it deposited in slender prisms that arranged themselves in clusters radiating from a common center. It melted at 127° C. The yield was quantitative. Analysis (Kjeldahl):

0.1368 gm. of substance gave 0.02044 gm. of nitrogen = 14.6 c.c. $\frac{N}{16}$ HCl.

For $\text{C}_6\text{H}_5\text{N}_2\text{SI}$ —

Calculated: N = 14.94 per cent.

Found: N = 14.94 per cent.

When 5 grams of this amino-pyrimidin were boiled with concentrated hydrochloric acid for one hour mercaptan was evolved

and it was converted practically quantitatively into 5-iodo-cytosin. Analysis (Kjeldahl):

0.1386 gm. of substance gave 0.02422 gm. of nitrogen = 17.3 c.c. $\frac{N}{16}$ HCl.

For $C_4H_4ON_2I$ —

Calculated: N = 17.72 per cent.

Found: N = 17.47 per cent.

2-Ethylmercapto-5-iodo-6-anilino-pyrimidin, $C_{12}H_{12}N_2SI$, $\begin{array}{c} N=C.NHC_6H_5 \\ | \quad | \\ SC-Cl \\ || \quad || \\ N-CH \end{array}$

was obtained by warming a benzene solution of 2 grams of 2-ethylmercapto-5-iodo-6-chlorpyrimidin and 1.4 grams of aniline. After evaporating the excess of benzene and washing with water to dissolve aniline hydrochloride, the base was obtained as an oil which would not solidify. It was purified by converting it into its sulphuric acid salt. This crystallized from alcohol, which contained sulphuric acid, in well developed prisms. The sulphate had no definite melting point but decomposed above 200° C. A nitrogen determination (Kjeldahl) agreed with the calculated value for a normal sulphate:

0.0923 gm. of substance gave 0.00938 gm. of nitrogen = 6.7 c.c. $\frac{N}{16}$ HCl.

For $(C_{12}H_{12}N_2SI)_2H_2SO_4$ —

Calculated: N = 10.34 per cent.

Found: N = 10.17 per cent.

2-Anilino-6-oxypyrimidin, $C_8H_7NH.C \begin{array}{c} NH-CO \\ | \quad | \\ CH- \\ || \quad || \\ N-CH \end{array}$.— This compound

was prepared by warming on the steambath 2-ethylmercapto-6-oxypyrimidin with the calculated quantity of aniline. It was also formed when an alcoholic solution of the mercaptopyrimidin and aniline were digested for several hours. It was moderately soluble in alcohol, and insoluble in benzene and water. It was purified for analysis by crystallization from alcohol. It deposited in well developed plates and melted at 230°–231° C. to a yellow oil. Analysis:

For $C_{10}H_9ON_2$ —

Calculated: N = 22.46 per cent.

Found: N = 22.54 per cent.

Action of Aniline on 2-Ethylmercapto-5-iodo-6-oxypyrimidin.— Fifteen grams of the iodopyrimidin were heated on the steambath

with 25 grams of aniline for six hours. It dissolved in the warm aniline to give a clear solution which gradually assumed a dark blue color. After the evolution of mercaptan ceased, the excess of aniline was removed by distillation with steam. We obtained a dark crystalline product that was insoluble in water. It was washed with cold alcohol to remove the coloring matter and purified by repeated crystallizations from alcohol and acetic acid. It failed to respond to tests for iodine and sulphur. It melted at 230° – 231° C. to a yellow oil. It agreed in all its properties with 2-anilino-6-oxypyrimidin (M. P. 230° – 231° C.). A mixture of the two compounds melted sharply at 230° – 231° C. Analyses for carbon, hydrogen, and nitrogen gave the following results:

0.0845 gm. of substance gave 16.7 c.c. nitrogen gas at 20° C. and 768 mm.

0.2491 gm. of substance gave 0.1193 gm. of H_2O and 0.5881 gm. of CO_2 .
Calculated for $C_{10}H_8ON_2$ —

	Calculated for $C_{10}H_8ON_2$ —	Found—
C	64.2 per cent	64.4 per cent
H	4.8 " "	5.3 " "
N	22.46 " "	22.8 " "

Action of Aniline on 2-Ethylmercapto-5-iodo-6-aminopyrimidin.

—This pyrimidin was recovered unaltered after heating with aniline for nine hours at 100° C. It melted at 127° C. and when mixed with the original material the melting point was not lowered.

Action of Aniline on 5-Iodocytosin.—This base was recovered unaltered after heating with aniline, in benzene, for one hour at 150° – 160° C. The temperature was then raised to 190° – 193° C., and maintained for two hours. When the tube was examined there was no evidence that any reaction had taken place. The iodocytosin was again recovered unaltered.

Action of Aqueous Ammonia on 2-Ethylmercapto-5-iodo-6-oxypyrimidin.—Five grams of the pyrimidin were heated with concentrated aqueous ammonia for three hours at 156° to 163° C. When the tube was examined the solution had assumed a red color and drops of mercaptan were floating on the surface of the liquid. The solution gave a strong test for iodine. The ammoniacal solution was evaporated to dryness. We obtained a crystalline residue that was soluble in water except a small amount of white crystalline material. This was identified as

the unaltered 2-ethylmercapto-5-iodo-6-oxypyrimidin. It melted at 196°C . and gave a strong test for iodine. The aqueous filtrate was concentrated on the steambath and combined with a strong solution of picric acid. We obtained a beautiful yellow picrate that crystallized from hot water in prisms. It had no definite melting point but decomposed above 260°C . and effervesced violently at about 285°C . It did not contain water of crystallization. It contained neither iodine nor sulphur. A nitrogen determination agreed with the calculated value for 2-amino-6-oxypyrimidin (XIV) (Isocytosin). Analysis:

0.1229 gm. of substance gave 0.03052 gm. of nitrogen = $21.8\text{ c.c. } \frac{\text{N}}{10}\text{ HCl}$.

For $\text{C}_4\text{H}_5\text{ON}_3 \cdot \text{C}_6\text{H}_3\text{O}_7\text{N}_3$ —

Calculated: N = 24.70 per cent.

Found: N = 24.83 per cent.

Action of Alcoholic Ammonia on 2-Ethylmercapto-5-iodo-6-oxypyrimidin.—After heating the pyrimidin with alcoholic ammonia for six hours at 141° to 148°C . it was recovered unaltered. It was then heated for six hours at 150° to 155°C . Under these conditions only a trace of mercaptan was detected. The contents of the tube were evaporated to dryness. We obtained a crystalline residue. This was insoluble in water and after one crystallization from alcohol it melted at 195°C . It contained both sulphur and iodine. When mixed with the original 2-ethylmercapto-5-iodo-6-oxypyrimidin the melting point was not altered. We did not detect the presence of any isocytosin.

Action of Alcoholic Ammonia on 5-Iodocytosin.—Five grams of iodocytosin were heated with strong alcoholic ammonia for six hours at 170° to 180°C . When the tube was opened considerable decomposition had taken place, and a well crystallized product was suspended in the alcohol. It crystallized from hot water in plates. It did not melt below 300°C . and contained no iodine. It contained one molecule of water of crystallization and agreed in all its properties with *cytosin*. Analysis:

0.2711 gm. of substance lost 0.0372 gm. of H_2O at 110° – 120°C .

For $\text{C}_4\text{H}_5\text{ON}_3 \cdot \text{H}_2\text{O}$ —

Calculated: H_2O = 13.95 per cent.

Found: H_2O = 13.72 per cent.

0.0909 gm. of anhydrous base gave 0.0343 gm. of nitrogen = $24.5\text{ c.c. } \frac{\text{N}}{10}\text{ HCl}$.

For $C_4H_4ON_2$ —

Calculated: N = 37.84 per cent.

Found: N = 37.73 per cent.

Action of Aqueous and Alcoholic Ammonia on 5-Iodouracil.—

When iodouracil was heated with concentrated aqueous ammonia at 170° – 180° C. for four hours it was recovered unaltered. Some of the pyrimidin was then heated with strong alcoholic ammonia under the same conditions. When the tube was examined slight decomposition had taken place and a colorless compound was suspended in the alcohol. It crystallized from hot water in balls of microscopic prisms and did not melt below 300° C. More of the same material was obtained when the alcoholic solution was concentrated. It did not contain iodine and was identified as uracil. The yield was practically quantitative. We did not detect the presence of any amino-uracil. Analysis (Kjeldahl):

0.1192 gm. of substance gave 0.02982 gm. of nitrogen = 21.3 c.c. $\frac{N}{16}$ HCl.

0.0757 gm. of substance gave 0.0189 gm. of nitrogen = 13.5 c.c. $\frac{N}{16}$ HCl.

For $C_4H_4O_2N_2$ —

Calculated: N = 25.00 per cent.

Found: N = (1) 25.01; (2) 24.96 per cent.

α -Cyanbutyrylurea, $CH_3 \cdot CH_2 \cdot CH(CN) \cdot CO \cdot NH \cdot CO \cdot NH_2$.—

This compound was obtained by condensing α -cyanbutyric acid with urea. We proceeded according to the directions given by Traube¹ for the preparation of cyanacetylurea from cyanacetic acid and urea. It crystallized from water in arborescent crystals and melted at 181° C. with slight effervescence. Analysis:

For $C_6H_8O_2N_2$ —

Calculated: N = 27.1 per cent.

Found: N = 27.5 per cent.

2, 4-Dioxy-5-ethyl-6-aminopyrimidin, $\begin{array}{c} N=C \cdot NH_2 \\ | \quad | \\ CO \quad CHC_2H_5 \\ | \quad | \\ NH \quad CO \end{array}$ — α -Cyanbu-

tyrylurea dissolved in 33 per cent. sodium hydroxide solution with absorption of heat. Fourteen grams of the urea were dissolved in 42 grams of the alkali solution and allowed to stand for several hours at ordinary temperature. The solution was then neutralized with hydrochloric acid. We obtained a

¹ Ber. d. deutsch. chem. Gesellsch., xxxiii, p. 1371, 1900.

beautiful crystalline product that decomposed at 325° – 330° C. with violent effervescence. It crystallized from hot water in needle-like prisms and decomposed at 339° C. with effervescence (using an Anschutz thermometer). It was soluble in hydrochloric acid, and was reprecipitated unaltered with ammonia. Analysis:

For $C_5H_5O_2N_3$ —

Calculated: N = 27.1 per cent.

Found: N = 26.9 per cent.

DESCRIPTION OF PLATE I.

Figure 1. 5-Iodocytosin, crystallized from hot water. Magnified 60 times.

Figure 2. The acetic acid salt of 5-iodocytosin. Magnified 60 times.

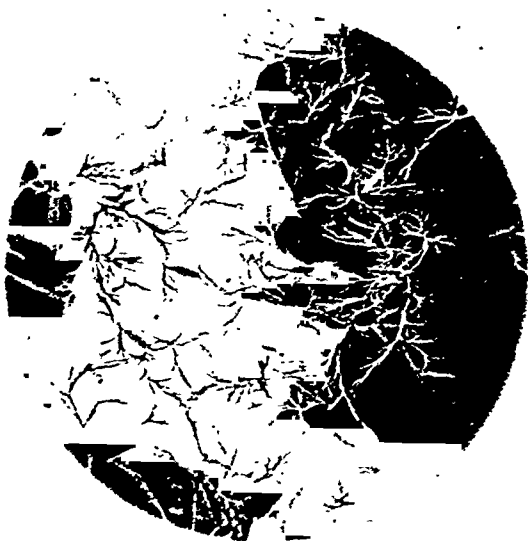


FIGURE 1.



FIGURE 2.

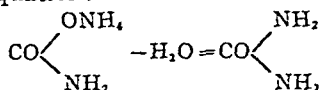
CONTRIBUTIONS TO OUR KNOWLEDGE OF THE CHEMISTRY OF CARBAMATES.

By J. J. R. MACLEOD AND H. D. HASKINS.¹

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A brief outline of the literature relating to the presence of carbamates in animal fluids, and in oxidation mixtures, has been given by us in a previous article and need not be repeated here.² Some of the workers referred to have claimed that urea is formed in the animal body by dehydration of ammonium carbamate according to the equation:



That carbamates are readily formed under certain chemical conditions, and that these conditions may exist in the animal body, is admitted in all the papers referred to, but that it is by a dehydration of this carbamate that urea is formed in the animal metabolism is not unanimously accepted.

The chief exponents of the hypothesis of the carbamate derivation of urea are Drechsel and Abel and Nencki and his co-workers, and its chief opposers are Hofmeister and Nolf.

For the detection of carbamates these investigators employed a method devised by Drechsel (*vide* previous paper), which, however, even when most carefully practised, could not be regarded as absolute, and which possessed no quantitative accuracy.

As the conversion of carbamate of ammonia into urea is a simple enough process in the chemical laboratory (dehydration by heating under pressure, and alternating oxidation and reduction) it is considered by the Drechsel school as of primary importance for the establishment of their hypothesis to show that carbamates actually exist in the animal body; it is

¹ H. M. Hanna Research Fellow.

² *Amer. Journ. Physiol.*, xii, p. 444, 1905.

considered that if they exist there can be little doubt that they are on the way to urea formation.

Under certain conditions (lime feeding, after the establishment of Eck's fistula, etc.), it has been found that the urine examined by Drechsel's method yields a considerable amount of carbamate, although, under normal conditions, by this method, such is not the case. Carbamates have further been found by Drechsel in the blood, and in the alkaline urine of herbivorous animals.

By the opponents of the hypothesis there is raised, as an argument against all these findings, the fact that in the employment of Drechsel's method, conditions are favorable for a formation of carbamates *de novo*, viz., that free carbonic anhydride and ammonia are readily produced from the carbonates and ammonia salts in the fluids examined, and that they will then combine to form carbamates (and carbonates).

This criticism has been satisfactorily answered by Abel¹ and others who have pointed out that the addition of excess of milk of lime to fluids containing ammonia salts and soluble carbonates will immediately fix the *free* carbonic anhydride present, as insoluble calcium carbonate, and at the same time drive off the ammonia.

On the other hand, it is evident that in simple mixtures of ammonia salts (*e. g.*, ammonium chloride) and soluble carbonates (*e. g.*, sodium carbonate), in which exist the ions NH_4^+ , Na^+ , Cl^- , CO_3^- , a certain amount of ammonium carbonate will necessarily be formed, and probably also some carbamate.

Before prosecuting our investigations further, therefore, we decided to re-investigate, by the use of the quantitative method for carbamate estimation previously described,² the question of the formation and stability of carbamates in such fluids, and then to see whether, when the urine was mixed with soluble carbonates, the ammonia salts which it contains would bring about the same reactions. We have considered that comparable results would best be obtained by estimating not only the carbon dioxide of carbamate, but also the total carbon dioxide and ammonia contained in the solution.

¹ *Johns Hopkins Hosp. Bull.*, No. 39, April, 1894.

² *Loc. cit.*

We have, therefore, expressed the amount of carbamate present in terms either of the ratio between carbamate carbon dioxide and total carbon dioxide or between carbamate ammonia and total ammonia. Certain of our investigations, bearing especially on the stability of carbamates in watery solution, have related to solutions of crystalline ammonium carbamate and the commercial carbonate.

These investigations are arranged under the following headings:

I. The carbamate content of watery solutions of various strengths of a preparation of crystalline ammonium carbamate that had stood some months under alcohol, and of freshly prepared ammonium carbamate.

II. The influence of standing at room temperature and at body temperature on the carbamate content of the above solutions.

III. The influence of adding excess of ammonia and of soluble carbonates on the carbamate content of the above solutions.

IV. The carbamate formation in solutions containing soluble carbonates and ammonium salts, and the formation of carbamate produced by adding soluble carbonates to urine.

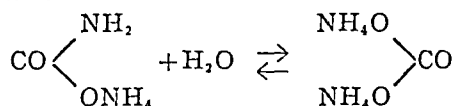
V. Since ammonium carbamate will partly dissociate into two ions in watery solutions and ammonium carbonate dissociates (partly at least) into three, we have investigated, by Beckmann's method, the depression of freezing point produced by solutions of ammonium carbamate before and after raising their temperature.

VI. Lastly we have appended a brief description of the method found by us most suitable for preparing pure calcium carbamate.

I. THE CARBAMATE CONTENT OF WATERY SOLUTIONS OF AMMONIUM CARBONATE AND CARBAMATE.

When ammonia and carbon dioxide come together carbamate of ammonia is formed, which is quite stable if kept from the presence of water, but soon becomes converted into ammonium carbonate and acid ammonium carbonate when water is present. This conversion of carbamates into carbonates in the presence of water does not proceed so far as to convert all the carbamates into carbonates but only until a certain equilibrium between

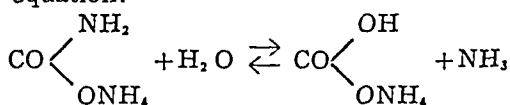
the two has been struck. The reaction may be represented by the following equation¹:



It seemed to us of interest to determine in what proportion or ratio the carbamate carbon dioxide stands to the total carbon dioxide after equilibrium has been established. As has already been pointed out, it is important to note that very different results regarding this ratio between total carbon dioxide and carbamate carbon dioxide have been obtained by us with solutions of crystalline ammonium carbamate and with solutions in which, by double chemical interchange between soluble carbonates and ammonia salts, ammonium carbamate might be formed. For the present we shall study *solutions of crystalline ammonium carbamate and carbonate*. For this purpose we dissolved ammonium carbamate (kept under alcohol) in water so as to make as nearly as possible a gram-molecular solution (*i.e.*, 7.8 per cent.) and made various dilutions of this in which we then determined, by the method discussed in our previous paper,² the relation of carbamate carbon dioxide to total carbon dioxide. The ammonia was also determined in these solutions by Folin's method.³ From these values was calculated the carbamate ammonia and its ratio to the total ammonia.

From determinations of the relative amounts of total carbon dioxide and total ammonia, it was found that ammonium carbamate, after standing some time (ten months) under alcohol, had a composition represented by the formula, $(\text{NH}_4)_2\text{CO}_3 + 2\text{NH}_4\text{HCO}_3$, which in chemical text-books is usually called *sesquicarbonate*. By standing, ammonia had gradually diffused away, the flask not being specially sealed. The estimations made on solutions of this preparation are marked A in the subjoined table.

¹ A certain amount of carbamate will be converted into acid carbonate according to the equation:



² *Loc. cit.*

³ *Zeitschr. f. Physiol. Chem.*, xxxvii, p. 161, 1903.

In freshly prepared ammonium carbamate, on the other hand, were found amounts of ammonia and carbon dioxide corresponding to the formula $(\text{NH}_4)_2\text{CO}_3$. The estimations on solutions of this preparation are marked B.

In both cases the carbamate of ammonia is included with the $(\text{NH}_4)_2\text{CO}_3$.

In columns 3 and 6 of the following table are given, in grams, the amounts of carbon dioxide and of ammonia in 100 c.c. of solutions of the above-mentioned preparations, and, in column 2, the calculated amount of carbon dioxide¹ for the above formulæ. In columns 4 and 7 of the same table are given the amounts of carbon dioxide and ammonia in carbamate, the former being directly determined, the latter calculated from the carbamate carbon dioxide. The relation of carbamate carbon dioxide to total carbon dioxide is given in column 5, and the ratio of carbamate ammonia to total ammonia in column 8.

TABLE I.

1	2	3	4	5	6	7	8
No. of solution.	Theoretical amount of CO_2 to satisfy the formula $2\text{NH}_3 + \text{HCO}_2 + (\text{NH}_4)_2\text{CO}_3$ per 100 c.c.	Grams CO_2 per 100 c.c.	Grams carbamate CO_2 per 100 c.c.	Carbamate CO_2 as per cent. of total CO_2 .	Grams NH_3 per 100 c.c.	Grams NH_3 in carbamate per 100 c.c. (calculated from carbamate CO_2).	Carbamate NH_3 as per cent. of total NH_3 .
A 3	0.330	0.330	0.020	6.08	0.17	0.0154	9.
A 14	0.465	0.441	0.027	6.3	0.24	0.021	8.7
A 6	0.442	0.473	0.029	6.17	0.228	0.0224	9.8
A 9	0.640	0.639	0.037	6.0	0.330	0.029	8.7
A 36	—	0.657	0.0698	10.7	—	—	—
A 18	0.90	0.875	0.1015	11.7	0.467	0.0784	16.8
A 2	—	1.650	0.160	10.3	0.85	0.123	14.4
A 1	—	3.310	0.430	13.2	1.70	0.332	19.4
	To satisfy formula $(\text{NH}_4)_2\text{CO}_3$						
B 77	0.966	0.952	0.210	22.2	0.748	0.162	21.6
B 79	0.538	0.5303	0.0679	12.9	0.416	0.0524	12.5
B 82	—	0.637	0.1077	17.	(6.500 calculated).	0.0832	16.6

It will be noted in connection with solutions of the preparation A that as the strength of solution increases the relative

¹ Calculated from the ammonia.

amount of carbamate becomes greater, gradually rising from 6 per cent. in the weakest solution examined to 13.2 per cent. in one ten times stronger. It will be further noted that in terms of ammonia the percentage of carbamate is greater than it is in terms of carbon dioxide. This apparent discrepancy is explained by the fact that in these solutions large amounts of acid ammonium carbonate (NH_4HCO_3) are present, so that the fraction which is not present as carbamate contains a relatively greater amount of carbon dioxide (in relation to the ammonia) than does the fraction present as carbamate. In the solutions marked B both ratios agree pretty closely. In these solutions, the fraction not included as carbamate yet contains the same ratio of carbon dioxide to ammonia as does carbamate. In the case of the solutions marked B (*i. e.*, of freshly made ammonium carbamate) the relative amounts of carbamate are also much higher than in A.

We do not suppose that the values for carbamate here depicted represent the amounts actually contained in the solution, some decomposition of carbamate being possible during the manipulations, but, inasmuch as the solutions were all examined by exactly the same procedure, their relative values are probably accurate enough, and they answer our purpose, *viz.*, to serve as standards for our further work.

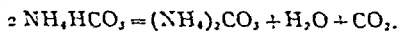
II. THE INFLUENCE OF STANDING ON THE CARBAMATE CONTENT OF THE ABOVE SOLUTIONS.

It is commonly stated that when solutions containing carbamate are allowed to stand the relative amount of carbamate diminishes. This we have found to be true only when the solutions are placed in open vessels. When however the solutions are contained in perfectly closed flasks and, especially if these be placed in the incubator at 36°C. , the relative amount of carbamate in the solutions may increase instead of decrease. In the following table (II), the total carbon dioxide and carbamate carbon dioxide in 1 c.c. of solution are given according to the reading (in millimeters) in the Barcroft-Haldane apparatus. The percentage of carbamate carbon dioxide to total carbon dioxide is also given. In brackets, after certain of the figures, are given corresponding readings on the same solutions immediately after making.

TABLE II.

1 No. of solution.	2 Condition of solution.	3 Millimeters of CO ₂ in 1 c.c.	4 Millimeters of carbam.-CO ₂ in 1 c.c.	5 Carbam.-CO ₂ in per cent. of total CO ₂ .
32	Solution stood in incubator 48 hours.	1100 [1300]	250 [1575]	22.7 [11.7]
40	In incubator 2 days; then 1 day at room temperature.	1260 [1300]	290 [1575]	23 [11.7]
62 urine	Stood at room temp. over night.	178.7	38 [24]	21.3 [13.3]
65 urine	Stood at room temp. several hours.	234	42 [26]	16.4 [11.1]
66a	In loosely cork- ed flask in in- cubator 3 days.	1337.5 [1400]	200 [160]	15 [11.7]
66b	In very tightly closed flask in incubator 3 days.	1400 [1400]	267.5 [160]	19 [11.7]
70a	In loosely cork- ed flask in in- cubator 1 day.	742 [858]	90 [90]	12.1 [12.3]
70b	In tightly cork- ed flask in in- cubator 1 day.	858 [858]	168 [95]	19.5 [12.3]
78		1300 [1460]	370 [325]	26.9 [22.2]

It will be observed that in most of these solutions the total carbon dioxide per cubic centimeter had diminished slightly, showing that some carbon dioxide had escaped from the flask. The ammonia, however, remained unchanged in amount. It might therefore be supposed that neutral carbonate, and with it carbamate, had become formed at the expense of the acid carbonate, thus:



This spontaneous evolution of carbon dioxide is known to occur at body temperature, 36° C.¹ That this formation of neutral carbonate is not the cause of the increase of carbamate is, however, shown in the observations marked 66 and 70 in the above table. In flasks *a* and *b* in each of these cases equal portions of

¹ Prescott and Johnson, *Qualitative Chemical Analysis*, 1891, p. 185.

the same solution of ammonium carbamate were placed, the flask containing portion *a* being loosely corked, that containing *b* hermetically corked. It was found, after incubating, that the ammonia did not diminish in amount in either solution, but that carbon dioxide escaped from the loosely corked flask. Had the above explanation been correct more carbamate would have been expected in *b* than in *a*, whereas much more was found in *a* than in *b*.

It would appear, therefore, that in ammonium carbonate solutions a temperature equal to that of the animal body increases, instead of diminishes, the relative amount of carbamate when the solution is kept in an hermetically sealed vessel. What the cause of this increase may be we are not prepared to state.

III. THE ADDITION OF SOLUBLE CARBONATES AND OF AMMONIA WATER TO THE ABOVE SOLUTIONS.

The relationship of carbamate carbon dioxide to total carbon dioxide in solutions of ammonium carbamate can also be caused to vary by changing the relative amounts of carbon dioxide and ammonia in the solution.

A.—When ammonia is present in excess of that represented by the formula $2\text{NH}_4\text{HCO}_3 + (\text{NH}_4)_2\text{CO}_3$, the amount of carbamate carbon dioxide in relation to the total carbon dioxide markedly increases, as has already been pointed out in connection with Table I. Thus in a solution of commercial ammonium carbonate containing an excess of ammonia (over that in the above solutions) (A 16 and 17, Table III), and in solutions of the preparation marked A, to which ammonia water has been added (A 20-23), the following values were found:

TABLE III.

¹ No. of solution.	² Grams CO_2 per 100 c.c.	³ Grams carbam.- CO_2 per 100 c.c.	⁴ Carbam.- CO_2 in per cent. of total CO_2 .	⁵ Grams NH_3 per 100 c. c.	⁶ Grams carbam.- NH_3 per 100 c.c.	⁷ Carbam.- NH_3 in per cent. of total NH_3	⁸ Excess NH_3 over formula.
A 20	0.823	0.106	12.2	0.513	0.0819	15.9	0.089
A 22	0.843	0.1147	13.7	0.544	0.0886	16.2	0.110
A 21	0.823	0.127	14.2	0.5627	0.0981	17.4	0.1387
A 23	0.876	0.1487	17	0.610	0.115	18.8	0.159
A 16	0.848	0.170	20	0.537	0.131	24.4	0.0991
A 17	0.482	0.068	14.1	0.320	0.053	16.5	0.072

In the solutions of the preparation marked A with added ammonia (A 20-23, Table III) it is seen that as the ammonia increases (*vide* column 8) the amount of carbamate steadily rises (column 3). In No. 23, however, there is also a greater amount of total carbonate which may partly account for the increase in carbamate in this case. In the solutions of commercial ammonium carbonate (16 and 17) the excess of ammonia over that required to form sesquicarbonate is no greater than that in solutions 20-23, and yet the proportion of carbamate is higher. This is explained by the fact that the excess of ammonia in solutions of the commercial salt is combined with carbon dioxide as ammonium carbonate (*i.e.*, it indicates that these solutions contain relatively more neutral carbonate and, therefore, carbamate), whereas in the solutions of sesquicarbonate only a small part of the added ammonia will react with acid carbonate to form the neutral carbonate and carbamate.

B.—When soluble carbonates (sodium carbonate) are added to the solution it would be expected that the relative amount of carbamate carbon dioxide to total carbon dioxide would decrease. This is, however, not always the case, at least when moderate quantities of soluble carbonate are added. In such cases the carbamate carbon dioxide either remains practically unchanged or *increases* in amount. This increase is probably due to the sodium ions having replaced ammonia in some of the acid carbonate, the ammonia thus liberated then reacting with more acid carbonate to produce neutral carbonate and consequently carbamate.

In Table IV, from which these conclusions are drawn, the total carbon dioxide and carbamate carbon dioxide per cubic centimeter of solution are given in millimeters as read on the manometers of the Barcroft-Haldane apparatus.

IV. CARBAMATE FORMATION IN SOLUTIONS CONTAINING SOLUBLE CARBONATES AND AMMONIUM SALTS.

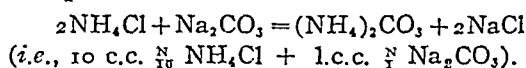
The above observations apply to solutions of crystalline ammonium carbamate and sesquicarbonate. Of more interest from a bio-chemical standpoint, however, is the study of the behavior of carbamate carbon dioxide in solutions containing soluble carbonates and ammonium salts. In solution in the blood

there are large amounts of soluble carbonates along with smaller amounts of ammonium salts. In the urine under certain conditions, such as after the ingestion of large quantities of lime (Abel) and after citrate administration, similar conditions exist. In such solutions complete dissociation of both salts (ammonium and carbonates) will occur and the ammonia and carbon dioxide will come together to form ammonium carbonate and carbamate.

TABLE IV.

No. of solution.	Nature of solution.	Total CO ₂ .	CO ₂ added in Na ₂ CO ₃ .	Carbamate CO ₂ .	Carbamate CO ₂ in per cent. of total CO ₂	Carbamate CO ₂ in per cent of CO ₂
24	10 c.c. ($\frac{N}{8}$) solution A + 10 c.c. $\frac{N}{16}$ Na ₂ CO ₃	660mm.	165mm.	7.33mm.	41.1 [6.08]	14.7
25	1 c.c. ($\frac{N}{8}$) solution A + 4 c.c. $\frac{N}{16}$ Na ₂ CO ₃	1345 "	66 "	100 "	7.4 [10.7]	7.7
37	1 c.c. ($\frac{N}{8}$) solution A + 4 c.c. $\frac{N}{16}$ Na ₂ CO ₃	1367 "	66 "	112.5 "	8.2 [10.7]	8.6
39	1 c.c. ($\frac{N}{8}$) solution A + 6 c.c. $\frac{N}{16}$ Na ₂ CO ₃	972 "	47 "	57 "	5.8 [11.17]	6.1
71	4 c.c. ($\frac{N}{8}$) solution A + 4 c.c. $\frac{N}{16}$ Na ₂ CO ₃	778 "	165 "	68 "	8.7 [7.3]	11.0

Taking first of all a solution containing a mixture of ammonium chloride and sodium carbonate in such proportion as to satisfy the equation:



in the subjoined table (V) numbers 64 and 67 give the results obtained with a mixture of 10 c.c. of $\frac{N}{16}$ ammonium chloride and 1 c.c. of $\frac{N}{8}$ sodium carbonate.

A large excess of carbamate carbon dioxide over that found in solutions of the sesquicarbonate is noted and it will be seen that in relation to the total carbon dioxide the carbamate carbon dioxide is markedly greater than in any of the solutions hitherto examined (compare with Table I). Exactly similar results were obtained with urine (human) to which sodium carbonate had been added just in sufficient amount to combine with the ammonia salts (Nos. 60, 61, and 63). To estimate how much sodium carbonate had to be added for this purpose it was neces-

sary first of all to determine the ammonia content of the urine, and also how much of the carbon dioxide of the added carbonate would be liberated by the acids of the urine, *i. e.*, how much of the sodium carbonate would be neutralized by the urine. For this latter purpose, a sample of the urine was mixed with a known quantity of sodium carbonate, the mixture shaken some minutes, and then the carbon dioxide determined in a measured quantity of it; the difference between the amount of this found and the amount which would have been found had the sodium carbonate solution been diluted with water instead of urine representing the sodium carbonate decomposed by the acid of the urine.

For example, of a sample of human urine, 25 c.c. gave in Folin's apparatus 18.6 c.c. of $\frac{N}{10}$ ammonia, therefore, 1.86 c.c. $\frac{N}{1}$ sodium carbonate is necessary to react with the ammonium salts in 25 c.c. of urine.

On mixing 1 c.c. $\frac{N}{1}$ sodium carbonate solution with 25 c.c. urine and shaking, it was found that only four-fifths of the carbon dioxide was recovered; therefore 0.2 c.c. $\frac{N}{1}$ sodium carbonate is necessary to neutralize the acid, or in other words:

$$1.86 \text{ c.c.} + 0.20 = 2.06 \text{ c.c.}$$

In Nos. 60, 61, and 63 of Table V are given the results of three observations of this nature.

Just as in solutions of ammonium chloride and sodium carbonate, the relative amount of carbamate as compared with solutions of crystalline carbamate is seen to be very high.

TABLE V.

No. of solution.	Grams CO_2 in 100 c.c.	Grams carbamate CO_2 in 100 c.c.	Carbamate CO_2 in per cent. of total CO_2 .	Grams NH_3 in 100 c.c.	NH_3 of carbamate.	Carbamate NH_3 in per cent. of total NH_3 .
60	0.149	0.0427	28.6	0.130	0.0330	25.3
61	0.117	0.0155	13.2	0.1075	0.0122	11.3
63	0.1585	0.0168	10.6	0.119	0.0130	10.9
64	0.2114	0.0297	13.8	0.1515	0.0229	15.0
67	0.186	0.0363	19.5	0.1515	0.0280	18.4

Regarding the relation of carbamate ammonia to total ammonia it will further be noted that this is practically the same as that of the carbamate carbon dioxide to the total carbon dioxide. In the solutions in question there is sufficient am-

monia to satisfy the equation $(\text{NH}_4)_2\text{CO}_3$ and, consequently, the fraction of solution which is not carbamate contains a relationship of ammonia to carbon dioxide similar to that in the carbamate fraction (cf. p. 324).

By standing in tightly corked flasks the carbamate does not become less in these solutions, indeed, as shown in Nos. 62 and 65 (Table II), the relative amount may become greater (without there being any fermentation of urea).

The addition of excess of sodium carbonate to these solutions decreases the relationship of carbamate carbon dioxide to total carbon dioxide. This is as might be expected since there is no acid carbonate in the solution to generate fresh ammonia by reaction with sodium ions (cf. p. 327).

TABLE VI.

No. of solution.	Nature of solution.	Total CO_2 in 1 c.c.	CO_2 added in excess of that necessary for reaction.	Carbam.- CO_2 in millimeters.	Carbam.- CO_2 in per cent. total CO_2 .	Carbam.- CO_2 in per cent. of CO_2 necessary for reaction.
67	10 c. c. $\frac{N}{10} \text{NH}_4 \text{Cl}$ + 1 c. c. $\frac{N}{1} \text{Na}_2 \text{CO}_3$	285 mm.		56	19.	19.6
68	10 c. c. $\frac{N}{10} \text{NH}_4 \text{Cl}$ + 1.25 c. c. $\frac{N}{1} \text{Na}_2 \text{CO}_3$	354 "	69 mm.	33	9.3	11.5
69	10 c. c. $\frac{N}{10} \text{NH}_4 \text{Cl}$ + 2 c. c. $\frac{N}{1} \text{Na}_2 \text{CO}_3$	530 "	245 "	32	6.03	11.2

Exactly the same formation of carbamate can be induced by causing carbonates to be excreted in the urine, as by the ingestion of citrates. Large amounts of carbamate are also contained in stale ammoniacal urine. The subjoined Table (VII) gives the results of our investigations relating to this.

All the urines included in this table were strongly alkaline towards litmus and effervesced on the addition of acid. Those marked J. J. R. M. contained a heavy deposit of phosphates.

Determinations were made of the total and carbamate carbon dioxide per cubic centimeter and the relationship between carbamate carbon dioxide and total carbon dioxide calculated.

The ammonia content was also determined by Folin's method and found extremely small in all the citrate urines. We with-

TABLE VII.

Nature of urine.	Grams CO ₂ per 100 c.c.	Grams carbamate CO ₂ per 100 c.c.	Carbamate CO ₂ in per cent. of total CO ₂	Remarks.
Citrate (dog's)	0.0791	0.0196	24.6	Contained 0.209 gr. NH ₃ in excess of that required to form carbamate.
" (J.J.R.M.)	0.0554	0.0058	10.5	
" (J.J.R.M.)	0.403	0.0543	13.5	Very small amount of ammonia.
" (J.J.R.M.)	0.2726	0.0336	12.4	Very small amount of ammonia.
" (J.J.R.M.)	0.4761	0.0491	10.4	
" (H.D.H.)	0.733	0.0516	7	Very small amount of ammonia.
Stale ammoniacal urine	1.219	0.375	30	Contained 0.32 gr. NH ₃ in excess of that necessary to form carbamate.

hold the results for the present until we have obtained more controls. This extreme reduction in the ammonia content of the urine after citrate ingestion has been noted by Burchard.¹ It also occurs after alkaline carbonate and lime feeding.

The table shows that in all the urines carbamate was present, and that it bore a fairly constant proportion to the total carbon dioxide. In the stale ammoniacal urine a very large amount of carbamate was found, the ammonia in this case being considerably in excess of the carbonic acid. When ammonium chloride is taken as well as citrates, the amount of carbamate in the urine is still greater.

We have controlled this observation by Abel and Drechsel's method and obtained a final precipitate which dissolved completely in ice-cold water, the solution depositing calcium carbonate on standing in a closed test tube at room temperature and evolving ammonia when gently warmed. In the Barcroft-Haldane apparatus it gave carbon dioxide with acid.

These results leave no doubt that carbamate is present in the urine whenever the latter contains soluble carbonates. As to whether the carbam-ion may pass through the renal filter as such or whether, when present in the urine, carbamates must

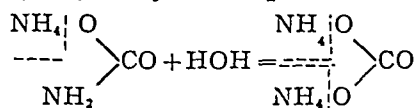
¹ Burchard, Inaug. Dissert. (Dorpat), 1889. Vide *Digest of Metabolism: Experiments*, Atwater and Langworthy, 1898.

be considered merely as the result of the above reaction remains an open question. We have never observed carbamate in the urine unless the latter was alkaline and contained a considerable amount of carbon dioxide. We consider that for the present the more important questions to settle in this connection concern the presence of carbonate in the urine, and with this investigation we hope immediately to proceed. It is evident from the above results that a certain amount of carbamate of ammonia must be present in the blood which is most probably converted into urea, but the question as to whether all the urea is formed in this way cannot be answered.

V. THE MOLECULAR CONCENTRATION OF FRESH AND WARMED SOLUTIONS CONTAINING CARBAMATE OF AMMONIUM.

To precipitate the soluble carbonates and bicarbonates for the purpose of estimating the carbamate the reagent employed by us is a mixture of barium hydrate, barium chloride, and ammonia. This reagent is added in large excess and the mixture shaken for half an hour.

Since this reagent does not precipitate as barium carbonate the carbon dioxide which is bound up in the carbamate, it suggests that the carbamic acid radical exists in the solution as a complex ion, $\text{NH}_2 \begin{array}{c} \text{O} \\ \diagup \\ \text{CO} \end{array}$. This being so it must follow that a solution of ammonium carbamate in ice-water contains fewer ions than the same solution after warming, for, by warming, the carbam-ions will be converted into ions of ammonium (NH_4) and carbonic acid (CO_3). By the equation:



two ions will have given place to three. The freezing point of water should, therefore, be found depressed to a less extent by a freshly prepared solution of ammonia carbamate (in ice-cold water) than by the same solution after it has been allowed to stand (tightly stoppered) for some time at a higher temperature. Such we have found actually to be the case as the following results show:

TABLE VIII.

CHANGES IN Δ (CORRECTED FOR SUPERCOOLING) PRODUCED IN SOLUTIONS CONTAINING AMMONIUM CARBAMATE BY RAISING THE TEMPERATURE (AVERAGE OF AT LEAST 3 READINGS IN EACH CASE).

Nature of preparation used.	Amount of CO ₂ in grams per 1 c.c. solution.	Δ before warming.	Δ after warming.	Difference in Δ produced by warming.
1. Sesquicarbonate (Prep. A).	0.0215	2.064	2.108	0.044
2. Sesquicarbonate (Prep. A) (several days after).	0.0215	2.063	2.102	0.039
3. Sesquicarbonate (Prep. A).	0.00559	0.556	0.585	0.029
4. Freshly made ammonium carbamate (Prep. B).	0.0172	2.060	2.385	0.325

The change thus produced in the molecular concentration of freshly made ammonium carbamate is relatively great.

In solutions of the sesquicarbonate (Prep. A) the difference though distinct and constant (for we have repeated the determination several times) is not great. This is undoubtedly due to the fact that all the carbamate is not converted into neutral carbonate, $(\text{NH}_4)_2\text{CO}_3$, but also into the acid carbonate, NH_4HCO_3 , which on dissociation will produce only two ions¹ so that in this part of the process of hydrolysis no increase in ionic concentration will occur.

Dilution of the solution of ammonium carbamate, as might be expected, increases the dissociation so that the increase of Δ produced by raising the temperature is still more marked (No. 3).

Conclusions.

1. Solutions of ammonium carbamate in water quickly decompose until a certain equilibrium between carbamate carbon dioxide and total carbon dioxide and between carbamate ammonia and total ammonia is established.

2. Solutions of ammonium carbonate (sesquicarbonate) contain proportionately less carbamate than the above because of the acid carbonate which they contain.

3. On standing for some time in a tightly corked flask the

¹Walker, *Introduction to Physical Chemistry*, 1901, p. 292.

carbamate does not become less in amount. In fact it appears slightly to increase.

4. The addition of ammonia to solutions of ammonium carbamate or carbonate increases the carbamate content.

5. The addition of small amounts of sodium carbonate to solutions containing the acid carbonate increases their carbamate content. The addition of excess of carbonate of course lowers the ratio of carbamate carbon dioxide to total carbon dioxide.

6. The addition of sodium carbonate to fluids such as urine which contain ammonium salts causes carbamate to be formed in relatively large amount.

7. Therefore stale urine and citrate urine contain carbamates.

8. A solution of ammonium carbamate, in ice-cold water depresses the freezing point of water to a distinctly less extent than does the same solution after standing a few minutes at room or body temperature. This is accounted for by the conversion of carbamate (two ions) into ammonium carbonate (three ions).

VI. A RAPID METHOD FOR THE PREPARATION OF PURE CALCIUM CARBAMATE.

About 100 c.c. of milk of lime, cooled to about 5° C., is placed in a large Florence flask and about 10 grams of ammonium carbamate, dried between filter paper, added. The mixture is vigorously shaken for about 15 minutes, being meanwhile kept cool by means of ice. After this time a few drops of a 10 per cent. solution of calcium chloride and a few crystals of calcium carbonate are added and the mixture again shaken.

The contents of the flask are removed to large centrifuge tubes previously cooled in ice, and centrifugalized.

The clear supernatant fluid is then filtered through a folded filter into an excess (six volumes) of 95 per cent. alcohol cooled to about 5° C.

When all has filtered through and the precipitate in alcohol has settled the precipitate is collected on a suction filter plate in a funnel surrounded by ice, quickly washed three times with cooled alcohol and twice with ether. It is then transferred to a vacuum desiccator.

To ascertain the purity of the preparation, a minute quantity (0.007 gr.) was dissolved in 25 c.c. of ice-cold water from which all the dissolved carbon dioxide had been expelled by boiling and of the resulting clear solution two samples of 3 c.c. each were mixed with acid in the Barcroft-Haldane apparatus. It was found that 0.241 c.c. and 0.244 c.c. of carbon dioxide gas were obtained. For the formula, $\text{Ca}(\text{NH}_2\text{CO}_2)_2$, 0.246 c.c. of gas should be derived.

FACTORS INFLUENCING SECRETION.

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The following is an attempt to make clear as far as possible the influences which control the secretory activities of the various glands of the body. It is certain that these influences are not the same in all glands, and a physiological grouping of the glands may be made, based on the means by which their activity may be stimulated or suppressed. The influence of changes in blood pressure and in the quantity of blood flowing through the organ is in some cases undoubted, but it will be seen that in a large number of glands this factor seems to play no rôle whatever. In the same way various substances influence the activity of some glands and not that of others. It will also be seen that muscular activity and the activity of certain glands may be influenced by the same factors, and seem to have something in common.

I. SECRETORY ACTIVITY WHICH IS INDEPENDENT OF BLOOD PRESSURE.

As described in previous papers,¹ a definite increase in the secretion into the intestines may be caused in a small fresh-water crustacean (*Sida crystallina*) by the administration of various chemicals, notably the ordinary purgatives and also barium chloride, pilocarpin, etc. These substances in solution are simply added to the water in which the crustaceans lie, and within a few moments not only increased peristalsis, but also increased secretion into the intestine takes place. It was further pointed out that in this animal there exists no closed blood-vascular system, the blood flowing within wide limits in more or less definite directions. There can therefore exist here nothing comparable with the blood pressure of vertebrates. In other

¹ J. B. MacCallum, *Amer. Jour. of Physiol.*, x, p. 101, 1903; x, p. 259, 1904. *Pflüger's Arch. f. d. ges. Physiol.*, civ, p. 421, 1904; *Univ. of Cal. Pub., Physiol.*, ii., p. 65, 1905.

words the secretion normally goes on in the intestinal and shell glands of this animal without blood pressure as a causative factor; and in addition to this, the secretion into the intestine may be markedly increased without an increase in blood pressure being possible.

Attention has already been called by Loeb¹ to the fact that in many organisms where a closed circulatory system does not exist, and where blood pressure can play no rôle, secretion certainly takes place.

In the slug (*Ariolimax columbianus*) there exists a fairly well-developed blood-vascular system, the capillaries being replaced largely, however, by a lacunar system. This animal is conspicuous for the abundant secretion which pours from its skin. This secretory activity may be stimulated and increased by numerous trifling causes. The skin is extremely sensitive to mechanical stimuli and to stimulation by various chemicals. I have made a number of experiments on this animal with solutions of various sorts. The local application of solutions of any of the saline purgatives to the skin causes abundant secretion. Barium chloride, sodium citrate, fluoride, sulphate, etc., in even very dilute solutions bring about almost immediately a well-marked secretory activity. This is not a mechanical stimulation, as water similarly applied has no such effect. If a few drops of one of these solutions be injected with a hypodermic syringe under the skin, the thick viscid secretion bursts out all over the body, and especially does it pour from the cavity of the mantle. Injection of water does not bring about this effect, nor does a solution of calcium chloride.

That this cutaneous secretion in the snail is independent of blood pressure, and indeed to some extent of the circulation of the blood, may be clearly shown. The beating of the heart may be seen distinctly just beneath the mantle on the floor of the mantle cavity. It is possible to remove this heart with sharp scissors without interfering seriously with the rest of the body. Although in the neighborhood of the wound the mechanical injury causes the skin to secrete, the more distant parts of the body surface remain at rest. If now a small quantity of a $\frac{1}{8}$ solution of sodium citrate or of one of the other purgative salts

¹ J. Loeb, *Science*, N. S., vii, p. 154; 1898.

mentioned above, be injected into the body, the same active secretion from the skin takes place as was the case in an animal from which the heart had not been removed. The secretion is, however, greater in the neighborhood of the injection, since the solution is not carried all over the body by the circulation as it is in the intact animal. If a few drops of $\frac{m}{8}$ barium chloride solution are placed on the skin surface of a slug from which the heart has been removed, a copious secretion appears in a way entirely similar to that described in the intact animal. There is at the same time a retraction of the skin under the solution, due probably to the contraction of the muscle fibres present under the skin. If a piece of the skin be cut entirely away from the slug's body, it still responds actively to stimulation by any one of the solutions mentioned, or to mechanical stimulation. If the animal be cut quickly in two with sharp scissors, the posterior half of the body behaves practically as it does in the normal animal with regard to chemical stimulation of the skin.

It is obvious from the above-described experiments that in the slug blood pressure cannot be looked upon as in any way a causative factor in the cutaneous secretion.

Secretion in loops of intestine removed from the body.—Results of experiments have already been published¹ which show that loops of the intestine of a rabbit, entirely removed from the body, may be made to secrete by immersing them in various solutions, particularly those containing barium chloride. The quantity of fluid secreted by such a loop was of course limited. Definite measurable quantities, however, were obtained when the loops were placed in sodium sulphate, fluoride, citrate, etc., or in barium chloride, while no secretion occurred in solutions of $\frac{m}{8}$ sodium chloride or $\frac{m}{8}$ calcium chloride. In other words, the same substances that cause increased intestinal secretion in the intact animal also produce the same result in loops of intestine removed from the body. In this instance it is not possible that blood pressure can have any influence in causing or modifying the secretion.

Action of pilocarpin on salivary secretion and on blood pressure.—It is well known that pilocarpin causes a very marked secretion

¹ J. B. MacCallum, *University of California Publications, Physiology*, i, p. 115, 1904.

from the salivary gland. It is also known that pilocarpin causes no rise in general blood pressure. Usually on the contrary, it causes a fall in pressure because of its action on the heart. T. C. Burnett and I have recently made some tests of the action of pilocarpin on the salivary gland and simultaneously on the blood pressure in the carotid artery. The intravenous injection of one-half to one cubic centimeter of one per cent. pilocarpin into a rabbit causes a marked secretion from the salivary gland, so that five to ten cubic centimeters of saliva were secreted in each case. At the same time the blood pressure in the carotid remained practically unchanged; in some cases it fell slightly. Barium chloride on the other hand, even in maximal doses, causes a much less active secretion and sometimes none at all, although the blood pressure in the carotid is very considerably raised. There seems to be no doubt that these substances do not influence the salivary secretion by influencing the blood pressure.

From the experiments described above it seems certain that, in some glands at least, changes in blood pressure play no rôle in secretion. The number of instances illustrating this could be greatly increased, but those given are sufficient to show that other factors must be looked for. And some light may be thrown upon this question by considering certain analogies which exist between muscular activity and glandular activity.

II. INSTANCES IN WHICH MUSCULAR AND GLANDULAR ACTIVITIES ARE CONTROLLED BY THE SAME FACTORS.

In endeavoring to produce secretory activity in various glands, it becomes obvious that the conditions which bring about this activity also produce muscular contraction. Those substances which bring about secretion from various glands simultaneously produce muscular activity; especially in the smooth muscles. To illustrate this a number of instances will be cited.

In the first place, peristaltic movements in the intestine may be brought about by the administration of saline purgatives, and these same solutions were shown to produce also a secretion of fluid into the intestine.¹ These solutions were shown by Loeb to produce rhythmical activity in the voluntary muscles of a

¹ J. B. MacCallum, *loc. cit.*

frog, and this activity was suppressed by calcium chloride¹ and magnesium chloride. Similarly it was later shown that calcium chloride and magnesium chloride counteract the effect of saline purgatives in producing peristaltic movements. It also suppresses the secretory activity in the intestine. Barium chloride, which is perhaps the most powerful salt in producing intestinal secretion, also brings about more active peristaltic movements than any other salt. Pilocarpin produces both increased secretion and increased peristaltic activity in the intestine. Barium chloride produces a greater secretion in the intestine than pilocarpin does, while in the salivary gland pilocarpin is more powerful. Their action on the musculature of the intestine is very similar. It is also interesting to note that atropin quiets the muscular movements of the intestine, and is also conspicuous in inhibiting the secretory activity of the salivary gland.

Mention must also be made of the muscular activity in the intestine brought about by section of the splanchnic nerves or by pithing the animal. These movements are extremely active, and have been variously explained as due to the removal of inhibitory nervous influences, etc. When the splanchnics are cut there is at the same time an active secretion of fluid into the intestines as described by Moreau.² This was believed to be analogous with the paralytic secretion produced in the salivary gland by section of the chorda tympani.

A striking instance of the simultaneous production of muscular movement and secretion of fluid is seen in the glands of the skin and nictitating membrane of the frog. These glands are very easy to watch in the nictitating membrane. They have roughly the shape of a Florence flask opening to the surface by a short narrow duct. They are lined by epithelium of a glandular type and surrounded by a layer of smooth muscle cells arranged so that their ends converge towards the two poles of the gland. This muscular layer was first described by Hensche³ and has been frequently described by later writers. It was observed much earlier, however, that the glands possessed a power of con-

¹ J. Loeb. *Festschrift f. Fick*, 1890; *Pflüger's Arch. f. d. ges. Physiol.*, xci, p. 248, 1902.

² Moreau, *Centralbl. f. d. med. Wissensch.*, 1868, p. 209.

³ *Zeitschr. f. wissenschaft. Zoologie*, vii, p. 273, 1856.

traction and expansion; and Ascherson¹ described the periodic movements and changes in size which the glands ordinarily underwent. Engelmann² described these in greater detail and found that the glands could be caused to contract by various modes of stimulation, *e. g.*, mechanical irritation, electrical stimulation, and the application of various chemicals—carbon dioxide, hydrochloric acid, acetic acid, etc. In this contraction the gland lumen was obliterated and the secretion forced out. The gland cells changed in shape and became thicker and more irregular. Stricker and Spina³ suggested that the cells in the contraction of the gland forced out fluid and in expansion took up new fluid. Biedermann, however, thought that the changes in the form of the cells were passive and due to the contraction of the whole gland. According to Drasch⁴ the secretion is a continuous one, even when the nictitating membrane is removed from the body and receives no blood supply, and when no periodic changes in shape can be observed in the cells. It was shown by Drasch that pilocarpin causes an active secretion of fluid from these glands. It also brings about a rapid contraction of the gland, so that the lumen is quickly obliterated. I have made experiments with a number of chemicals and found that barium chloride especially was active in causing these glands to contract and the gland cells to change their shape. From being flat, thin cells lining the distended gland, they became irregularly cubical or cylindrical cells. The lumen of the gland disappeared and the whole gland was reduced to a small fraction of its volume. A similar but less marked result was obtained with sodium citrate, sodium fluoride, and sodium sulphate. There is some similarity in the arrangement of the gland cells and the muscle in these glands and in the intestine. In both cases a layer of glandular epithelium is surrounded by smooth muscle cells, and in both cases the muscle is caused to contract and a secretion is produced by the same agents, *e.g.*, pilocarpin, barium chloride, sodium citrate, sulphate, etc.

A further instance of the simultaneous production of muscular

¹ *Arch. f. Anat. u. Physiol.*, 1840, p. 15.

² *Pflüger's Arch. f. d. ges. Physiol.*, v, p. 498, 1872.

³ *Sitzungsber. d. k. Akad. d. Wissensch., Wien*, lxxx, p. 95, 1880, quoted by Drasch.

⁴ *Arch. f. Physiol.*, 1880, p. 96.

and glandular activity is seen in the slug described above. When any one of the solutions which were spoken of as producing a secretion from the skin was applied, there was an almost immediate contraction of the skin under the area moistened by the solution. This was due to a contraction of the muscle fibres situated immediately beneath the skin. The glands and the muscles were thus made active by the same condition.

It is therefore evident from what has been cited above that in certain glands—those of the intestine, the salivary glands, the cutaneous glands of the slug, the glands of the nictitating membrane of the frog, etc.—the secretion is not dependent on changes in the blood pressure, and is even independent to some extent of the circulation of blood; and that those chemical agents which stimulate the glands to greater activity do so, not by influencing the blood pressure, etc., but by changing some other factor. It is further clear that these agents act through a property of the cell which is closely related to muscular contraction, and that it is probably more than a coincidence that muscular activity and glandular activity are produced simultaneously in so many cases by the same conditions. It is perhaps too much to suggest, as Stricker and Spina¹ did, that a rhythmical contraction is produced in the protoplasm of the gland cell similar to that produced in the muscle; and how such a contraction could produce secretion is not clear without assuming a valvular structure in the cell, of which there is no evidence. The squeezing out of the secretion from the lumen of the gland by the contraction of a muscle sheath surrounding the alveoli can have little to do with the actual process of secretion. Such a muscle sheath exists in the salivary gland² and attention has been called to its possible action in forcing out the saliva, by Miss Hyde³ and by A. P. Mathews.⁴

Although the real nature of the mechanism of secretion in the glands described is not yet clear, it is necessary to call attention to the close relation which seems to exist between muscular activity and the activity of the glands, and to assume that there is somewhere a common factor in the two processes.

¹ *Loc. cit.*

² Kolossow, *Arch. f. mikroskop. Anat.*, lii, p. 1, 1898.

³ *Zeitschr. f. Biol.*, xxxv, p. 463, 1897.

⁴ *Amer. Journ. of Physiol.*, iv, p. 485, 1901.

III. SECRETION IN WHICH CHANGES IN BLOOD PRESSURE AND IN THE CIRCULATION OF THE BLOOD PLAY A RÔLE.

In comparison with the group of glands described above, it is interesting to note a secretion which is definitely influenced by the quantity of blood flowing through the gland, and is to some extent dependent on the height of the general blood pressure. When we consider the activity of the kidney we find that those conditions which increase the flow of urine are not usually the conditions which increase the secretion from the group of glands described above. The so-called diuretics are in general not the substances which increase secretion into the intestine or from the salivary gland or skin. Pilocarpin, which increases so markedly the flow of saliva, of intestinal juice, and of the skin secretion, tends to diminish the flow of urine.¹ Atropin, which stops the secretion from the salivary gland, intestine, etc., has little or no effect on the kidney. Sodium citrate, sulphate, fluoride, etc., act as diuretics only when injected with large quantities of fluid, and the plethora produced is the main factor in causing an increased flow of urine. In the intestine they increase the secretion when injected in small quantities or applied locally to the peritoneal surface. Cascara sagrada causes an increased secretion into the intestine, but is not known as a diuretic. Also many substances are active diuretics without at all affecting the intestine, salivary glands, skin, etc. Among these are digitalis, caffein, theosin, theobromin. An exception to this is barium chloride, which increases not only the intestinal secretion, but in minute doses also the flow of urine. In larger doses, however, it diminishes or stops the flow of urine.²

As is well known the flow of urine is distinctly influenced by the quantity of blood flowing through the kidney, as shown by the changes in the kidney volume measured by an oncometer. It is not in the same way influenced by changes in the general blood pressure, although there is a minimal blood pressure below which no secretion takes place. In the intestine, on the contrary, the blood pressure may be zero, *e.g.*, in the excised loop, and secretion still be possible.

¹ *University of California Publications*, ii, No. 13, 1905.

² *University of California Publications*, i, No. 10, 1904; *Journ. Exper. Zool.*, i, No. 1, 1904.

Of interest here also is the action of calcium and magnesium chlorides, which diminished not only the intestinal secretion, but also the secretion of urine.¹ This is difficult to explain, but two things suggest themselves in connection with it. There is in the kidney a large surface of epithelium in the convoluted tubules, which is of a type similar to that of the intestine, salivary gland, or cutaneous glands, *i.e.*, it is made up of columnar or cubical cells with a relatively large amount of protoplasm; and these cells are in striking contrast with the cells of Bowman's capsule. It is possible that these might be affected by agents which influence the cells of the intestine, *e.g.*, barium chloride, and calcium chloride.

Another possibility, however, may be spoken of. It was pointed out a short time ago² that diuresis and hæmolysis possess some factors in common. Certain powerful hæmolytics such as saponin and quillain were found to be also strong diuretics, while digitalis, which is well known as a diuretic, proved to have hæmolytic powers almost if not quite as strong as saponin. It was shown also that calcium and magnesium chlorides, which markedly decrease the flow of urine and prevent the action of most diuretics, inhibit also the hæmolytic action of saponin, etc. Thus the fact that these two processes (diuresis and hæmolysis) are controlled in certain cases by the same agents, suggests that the processes themselves may have something in common. In one case a fluid (the urine) passes out through the kidney cells, and this passage is facilitated by the administration of diuretics and retarded by calcium chloride and magnesium chloride. In the other case a fluid (hæmoglobin) passes out from the red blood corpuscles as the result of the administration of certain of these same substances which acted as diuretics, and this passage is retarded in the same way by calcium chloride. What most readily suggests itself is that the saponin, digitalin, etc., increase the permeability of the wall of the kidney cell and also that of the red blood cell; and that calcium chloride and magnesium chloride in both cases diminish this permeability. If this be true, changes in permeability must play a much more

¹ *Journ. of Exper. Zool.*, i, No. 1, 1904.

² J. B. MacCallum, *University of California Publications, Physiology* ii, No. 12, 1905.

important rôle in the action of diuretics than is ordinarily ascribed to them, and calcium chloride may owe its action both in the intestine and the kidney, in part at least, to its possible influence on the permeability of the cells.

That great changes in permeability do take place in the lumen side of certain gland cells during secretion is shown by such secretory activity as that of the sebaceous glands, mammary glands, and goblet cells of the intestine. In these cells the lumen side of the cell is either partially or wholly broken down when the gland is excited to secretion.

There are thus three main factors in secretion acting in various glands: in the first place an activity in the gland somewhat similar to muscular contraction and governed in many cases by the same conditions; secondly, the force which is exerted by a quantity of blood passing through the organ, the secretion diminishing or increasing according to the shrinking or swelling of the organ (kidney); and in the third place, in both of these classes of glands, changes in permeability play a rôle.

ON THE POLYMERIZATION OF GLOBULIN.

By ALONZO ENGLEBERT TAYLOR.

(From the Hearst Laboratory of Pathology, University of California.)

(Received for publication, January 14, 1906.)

Some eighteen months ago I prepared stocks of purified globulins from bovine serum. These globulins were prepared as follows: Filtered, diluted bovine serum was first precipitated by saturation with carbon dioxide in a closed system, the gas being held on overnight at a pressure of two feet of water. The precipitated globulin was collected upon a filter, washed with distilled water saturated with carbon dioxide, dissolved in water of a faint alkaline reaction, again precipitated by carbon dioxide, washed as before, and this process repeated a third time. This globulin was entirely insoluble in pure water; it was soluble in water in the presence of a trace of alkali or acid, except carbonic acid; it was precipitated from solution by dialysis, by saturation with magnesium sulphate, by half saturation with potassium acetate or ammonium sulphate; neutral salts had very little effect upon its solubility in water of neutral reaction. Ammonium sulphate gave the first distinct precipitation at about 30 per cent. saturation. When magnesium sulphate was employed for precipitation, the first distinct cloudiness appeared at about 60 per cent. saturation. This globulin corresponds to what Fuld and Spiro¹ have termed euglobulin, and to what Quinan² has termed insoluble globulin. This globulin was conserved by careful drying in a stream of dried air at 45° C., the last traces of water being removed by repeated washings with dried ether. The clumps were then powdered and washed with dried ether. The final product was a hornlike, granular powder that keeps indefinitely in a dry atmosphere. The second globulin was prepared as follows: The filtrate of the first precipitation of the diluted serum by carbon dioxide was precipitated by saturation with

¹ Fuld and Spiro, *Zeitschr. f. physiol. Chem.*, xxxi, p. 139, 1900.

² Quinan, *University of California Publications, Pathology*, i, p. 1, 1903.

magnesium sulphate. On the following day the precipitate was collected upon a filter, washed repeatedly with a saturated solution of magnesium sulphate, and then suspended in water, in which it dissolved. This solution was then filtered and the protein again precipitated by mixing it with slightly more than an equal volume of a saturated solution of ammonium sulphate. The precipitate was collected upon a filter and washed repeatedly with a half saturated solution of ammonium sulphate. The washed precipitate was then dissolved in water and the salt removed by dialyzation. This procedure of precipitation and dialyzation was again repeated. The collected globulin, after precipitation with alcohol-ether, was then washed rapidly with dried ether, several times extracted with ether, then dissolved in water and precipitated with ammonium sulphate. The treatment with ether was necessary to free it of lipoidal substances that were carried down with the precipitate. The final precipitate was then dissolved in the least possible amount of water, freed of salt by dialysis, and rapidly dried in a vacuum at 45° C, powdered, and repeatedly washed with ether. This powder is much finer and more crystal-like, less granular and horn-like, than the powder of the insoluble globulin; it is permanent on conservation in a dry atmosphere. This globulin corresponds to the pseudo-globulin of Fuld and Spiro,¹ to the soluble globulin of Quinan.² It was soluble in conductivity water; this solubility was not affected by the presence of acids, alkalies, or salts in low concentrations. It was not precipitated from solution by potassium acetate at half saturation. Precipitation by magnesium sulphate was not observed until the saturation of the salt had reached 80 per cent.; by ammonium sulphate, not until the saturation had reached 40 per cent. It was not precipitated from solution by carbon dioxide or by dialyzation.

The methods of preparation are not quantitative; there is a progressive loss of both globulins as they are purified. When conserved dry and sterile, these powdered globulins are permanent. I have recently tested preparations of the same age and found them unchanged. The amount of soluble globulin

¹ *Loc. cit.*

² *Loc. cit.*

in the blood serum is much greater than that of insoluble globulin

A few of the statements require comment. It is usually stated in the text-books that neutral salts aid the solution of the insoluble globulin. This is not true if the salts are neutral. The supposedly pure salts of many firms are not neutral. Solutions of the pure chlorides of sodium and potassium, and of the pure sulphates of sodium, potassium, and magnesium, are neutral in reaction when free of carbon dioxide. Solutions of ammonium sulphate and potassium acetate are always respectively acid and alkaline (when free of carbon dioxide), the result of hydrolytic dissociation. But many preparations of salts that should furnish neutral solutions present an acid or alkaline reaction, due to the inclusion of the mother solution from which they were crystallized. High concentrations of neutral salts aid the solution of insoluble globulin; low concentrations—such as one-half per cent.—have very little influence on the solubility. The method of washing a protein with dried ether has been found very advantageous for the purpose of freeing a protein of traces of water, without denaturation. The ether is best dried by the action of anhydrous sodium sulphate. This method of drying will not remove water of combination, it will not remove the last traces of water as completely as drying at 100° C., but it removes the water to such an extent that the powders are permanent, and accomplishes the result without any denaturation of the protein.

At the time these globulins were prepared, some of each was placed in distilled water in well-steamed bottles, conserved with a few drops of toluol, sealed and set aside. The temperature in the laboratory has ranged from 10° to 22° C. since that time. As time passed, I noticed that the contents of the bottle of soluble globulin, that was entirely clear when first prepared, exhibited a progressively increasing precipitation; while in the clear supernatant fluid over the white powder in the bottle of insoluble globulin, an opalescence appeared. The contents of both bottles were repeatedly shaken and mixed. I had set the tests aside with the intention of observing the occurrence of auto-hydrolysis. The appearances were therefore unanticipated, and recently the bottles were opened and the contents examined, a year and a half after they were first prepared.

The contents of the bottle of soluble globulin were first filtered and a notable amount of precipitate collected upon the filter. This precipitate gives the common tests for protein and the reactions of insoluble globulin. It is soluble in water to which a trace of alkali or acid has been added; it is insoluble in pure water; it is not aided in its solution by neutral salts at low concentration; it is precipitated from solution by carbon dioxide and by dialysis; it is precipitated from solution by half saturation with potassium acetate, by nine-tenths saturation with magnesium sulphate and by half saturation with ammonium sulphate. The soluble globulin in the filtrate exhibits the same properties it did when first placed in the bottle.

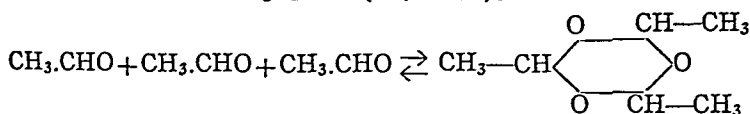
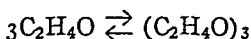
The contents of the bottle of insoluble globulin were filtered. The filtrate presented a faintly opalescent appearance and gave the common tests for protein. This filtrate contains a soluble globulin. It is soluble in conductivity water, is not precipitated by carbon dioxide or by dialysis, is not precipitated by half saturation with potassium acetate, and is precipitated by magnesium sulphate only at complete saturation. The residual insoluble globulin in the bottle presents the same properties it exhibited when first placed in the bottle. The observation that soluble globulin on standing in water becomes insoluble is not new; it has, however, been usually ascribed to a denaturation. The observation that insoluble globulin becomes soluble has not, to my knowledge, been described.

What has happened during the lapse of time is obvious. Soluble globulin in the one bottle has been converted into insoluble globulin, while in the other bottle insoluble globulin has been converted into soluble globulin. In the bottle containing originally insoluble globulin we have: insoluble globulin + water = insoluble globulin + soluble globulin + water; in the bottle containing originally soluble globulin we have: soluble globulin + water = soluble globulin + insoluble globulin + water. We have therefore the reversible reaction: soluble globulin \rightleftharpoons insoluble globulin. The reaction tends obviously to an equilibrium in the system. As stated, these globulins when conserved dry are permanent. Water is therefore necessary to the reaction. The contents of the bottles were sterile, the action of bacteria is therefore excluded. The influence of the toluol cannot be ruled

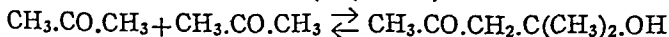
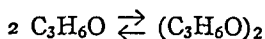
out, but there is nothing to indicate that it has been the active agent in the inauguration of the reaction. At the most, it could be expected to have acted only as a positive catalysor. The reaction must be ranged with the general group of reactions by polymerization.

Many of these reactions of polymerization are known. They tend to an equilibrium in the system, are therefore reversible and follow the law of mass action. Many of them are known to be susceptible of acceleration by positive catalysors. The relations may be elucidated by illustrations.

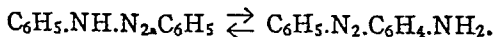
Aldehydes tend to form condensation products termed paraldehydes (Turbaba ¹). Thus acetic aldehyde passes into the corresponding paraldehyde.



Acetone passes into the isomeric di-acetone alcohol (Koelichen ²).



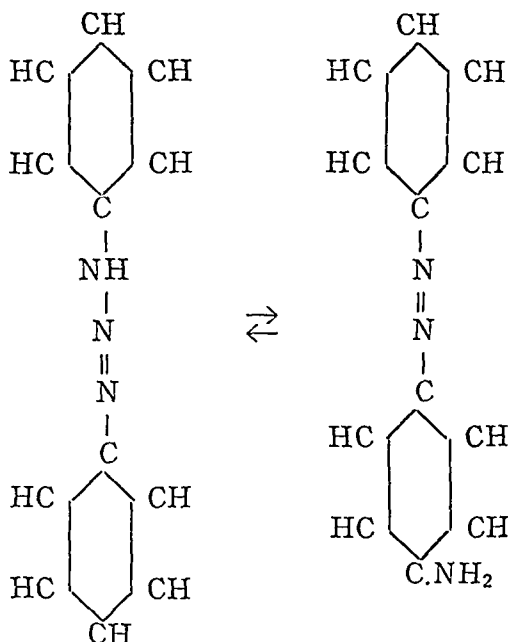
Diazoamidobenzol undergoes transformation into amidoazobenzol (Goldschmidt and Bardach, Goldschmidt and Reinders ³).



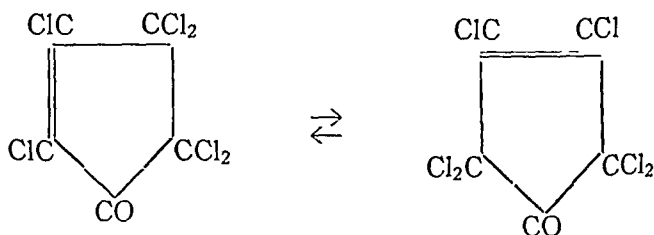
¹ Turbaba, *Zeitschr. f. physik. Chem.*, xxxviii, p. 505, 1901; *Zeitschr. f. Electrochem.*, viii, p. 70, 1902.

² Koelichen, *Zeitschr. f. physik. Chem.*, xxxii, p. 129, 1900.

³ Goldschmidt and Bardach, *Ber. d. deutsch. chem. Gesellsch.*, xxv, p. 1347, 1892; Goldschmidt and Reinders, *ibid.*, xxix, p. 1369, 1896.



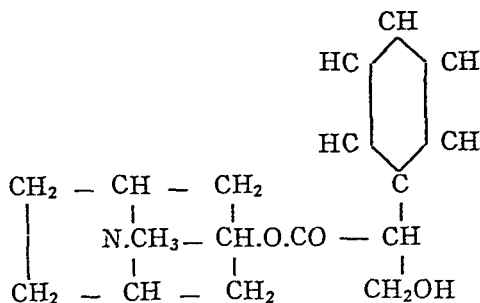
An interesting polymerization is afforded by the isomeric ketones, C_5Cl_6O (Zincke and Kuester¹).



The transformation of atropine into the isomeric hyoscyamine belongs to this class of reactions (Will and Bredig²). These substances are combinations of tropaic acid and tropine, and have in all probability the constitution:

¹ Zincke and Kuester, *Ber. d. deutsch. chem. Gesellsch.*, xxiii, pp. 187, 2212, 1890. Kuester, *Zeitschr. f. physik. Chem.*, xviii, p. 161, 1895.

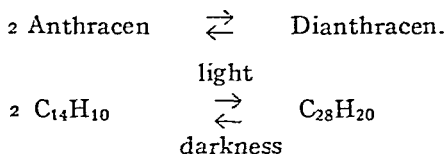
² Will and Bredig, *Ber. d. deutsch. chem. Gesellsch.*, xxi, p. 2777 1888



Atropine is an inactive substance, hyoscyamine polarizes light to the left. The hypothesis of Ladenburg¹ is that atropine is a racemic body and hyoscyamine is its l-component. Both the tropaic acid and the tropine contain asymmetric atoms of carbon, but nothing is known of the stereoisomeric configuration of these substances.

An exceedingly interesting illustration of this type of reactions is furnished by the conversion of anthracen into dianthracen, which has been studied especially by Orndorff and Cameron,² and Luther and Weigert.³

This is a reversible photochemic reaction (the chemical relations of which are not clear), which may be written as follows:



The dianthracen is relatively insoluble in phenetol, and when the reaction is carried out in this medium an amorphous suspension is produced by the reaction in the direction of the right, while during the reversed reaction this suspended substance passes into solution. This is directly analogous to the relations observed in the polymerization of globulin.

These examples are all illustrations of chemical equilibria. The following relations are common to all these reactions: The

¹ Ladenburg, *ibid.*, xxi, p. 3065, 1888 ; xxii, p. 2590, 1889.

² Orndorff and Cameron, *Amer. Chem Jour*, xvii, p. 658, 1895.

³ Luther and Weigert, *Zeitschr. f. physik. Chem*, li, p. 297, 1905.

velocity of reaction is a function of temperature. The velocity may be accelerated by the presence of positive catalysors. The reactions are reversible and there is a mass relation in the system. The station of equilibrium is specific to the particular system at constant temperature and is not translocated by the presence of a catalysor. With the possible exception of the reaction aldehyde \rightleftharpoons paraldehyde, these reactions are known to pass through intermediary stages. These different reactions have varying velocities at ordinary temperatures; none is, however, so slow as the globulin reaction. This has, however, no bearing upon the nature of the reaction; the velocity of a reaction does not indicate its quality.

This transformation of globulin might be either a physical or a chemical phenomenon. If it were a physical phenomenon, it would be comparable to the relations in sulphur. This substance, as is well known, may appear in two forms of crystallization: the rhombic or octahedral, and the prismatic or monosymmetric forms. This is a condition of physical equilibrium. Below 95.6° C. the rhombic crystals are formed; above that temperature the monosymmetric crystals are formed. At the temperature given, both forms are stable. Not only may the process, S-rhombic \rightleftharpoons S-monosymmetric, be made to proceed in one or the other direction by alterations in the temperature above or below 95.6° C., but it may be controlled also by pressure within the system. It is not probable that we are dealing with such a condition of physical equilibrium in globulin. It is quite characteristic of the physical equilibria that they have a sharp temperature factor, and that at the appropriate temperature the reaction proceeds with great rapidity. This was not the case in the instance of globulin. Furthermore, while in the case of physical transformations we may encounter marked deviations in physical properties, as in the melting-point, we do not usually encounter marked alterations in chemical relations, such as are to be seen in the instances adduced above and in the globulins. We may therefore conclude that we are dealing in all probability with a chemical and not a physical equilibrium.

Viewed as a chemical transformation, many possibilities are available. Of these, three seem the most probable. The

transformation might in the first place be considered as a condensation of two or more molecules, corresponding to the formation of paraldehyde from aldehyde, of dianthracen from anthracen. Thus globulin + globulin \rightleftharpoons globulin-globulin. The diglobulin would probably be assumed to be the insoluble form. This hypothesis could be tested directly by an estimation of the molecular weight, which is, however, at present impossible for this class of compounds.

Secondly, we might be dealing with a combination with water. Thus: globulin + water \rightleftharpoons globulin-water. Such a hypothetical equilibrium would be somewhat analogous to $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O} = \text{ZnSO}_4 \cdot 6\text{H}_2\text{O} + \text{H}_2\text{O}$. Upon this hypothesis the globulin-water would probably be the soluble form. That the reaction occurs only in the presence of water would not necessarily speak for this interpretation as against the other hypotheses, since water might be reasonably supposed to be necessary to the reaction, whatever the nature of the reaction might be.

The third hypothesis for the explanation of the reaction would lie in the assumption of an intramolecular re-arrangement, such as is noted in the reactions acetone \rightleftharpoons diacetone alcohol, diazoamidobenzol \rightleftharpoons amidoazobenzol, and the isomeric ketones $\text{C}_5\text{Cl}_6\text{O}$. This is made feasible through the assumption of the now currently conceded asymmetry of nitrogen. Under this hypothesis the process would be one analogous to a very common phenomenon in the domain of organic compounds.

Beyond this point there can be no profit in following the speculation. It is, however, of importance to realize the theoretical relations. The practical elucidation of the actual facts in the reaction cannot be hoped for until the constitution and molecular configuration of the protein are understood. That both forms of globulin occur in the blood serum suggests that the conditions there represent an equilibrium in the system and would therefore be an expression of the law of mass action. In a recent paper Moll¹ has described the formation of globulin from albumin and suggests the following cycle: albumin \rightarrow pseudo-globulin \rightarrow euglobulin \rightarrow alkali albuminate, from which the initial albumin could be again derived. These transforma-

¹ Moll, *Beitr. z. chem. Physiol. u. Path.*, iv, p. 563, 1903; vii, p. 311, 1905.

tions were described as occurring under the action of alkalies and proportional to the hydroxylion concentration and to the mass of albumin, *i. e.*, they were catalytically accelerated reactions. It is probable that the phenomena as described by Moll could be interpreted in a natural and unforced manner in accordance with the point of view here adopted. A somewhat analogous transformation of albumin into globulin has been suggested by the investigations of Sykes,¹ and it may be logically brought under the same point of view.

The original experiments were done to test the occurrence of auto-hydrolysis of globulin. The contents of both bottles presented evidences of the occurrence of a slow auto-hydrolysis. In both solutions a hydrated protein could be isolated, giving all the tests commonly ascribed to the peptone group—positive biuret reaction, non-precipitability by ammonium sulphate, precipitability by tannic acid and by iron. The substance was present in the largest amount in the bottle that contained originally soluble globulin. This fact and the general analogy suggest that it is the soluble globulin that undergoes the auto-hydrolysis. From this it follows that two simultaneous reactions occur in such solutions of globulin:

Soluble globulin \rightleftharpoons insoluble globulin.

Soluble globulin + water \rightleftharpoons proteose.

¹ Sykes, *Jour. Physiol.*, xxxiii, p. 101, 1905.

STUDIES ON THE BANANA.—I.

By E. MONROE BAILEY.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University.)

(Received for publication, December 26, 1905.)

The chemical changes which starch-containing fruits undergo incidental to their ripening processes have been the subject of numerous studies. Most of these, however, belong to a period when relatively little was known regarding the rôle of enzymes in physiological processes, and the significance of metabolic changes in plant tissues was scarcely appreciated. A useful review of the literature has been published by Gerber,¹ to whom various original contributions regarding a number of ripening fruits are due. He devoted particular attention to the respiratory processes concerned, in connection with the incidental changes in chemical composition. More recently the work of Stoklasa² and his collaborators, especially on the sugar beet, has been of particular value in pointing out the possible differences between aerobic and anaërobic respiratory exchange in relation to the stored carbohydrates of plant tissues, in directing attention to the fermentative changes which may be induced through the mechanism of intracellular enzymes like zymase and invertase, and in completely excluding bacterial changes from participation in all these processes. Lately the subject has received attention in a number of bulletins from the Bureau of Chemistry at Washington.³

The peculiar composition of the banana (*Musa*) and the readiness and rapidity with which it ripens especially recommend this fruit for the investigation of the processes here involved. Buignet⁴ long ago assumed that a difference exists between

¹ Gerber, *Annales des sciences naturelles*, Series VIII, p. 4, 1896.

² Stoklasa, Jelinek, and Vitek, *Beiträge zur chemischen Physiologie und Pathologie*, iii, p. 460, 1903.

³ United States Department of Agriculture, Bureau of Chemistry, Bulletin 87, 1904; Bulletins 94 and 97, 1905.

⁴ Buignet, *Comptes Rendus*, xlix, p. 276, 1859.

sugars of fruits like the banana according as they are ripened after picking or allowed to undergo ripening on the tree, more sucrose and less invert sugar being present in the latter case. Corenwinder¹ later studied the changes in the proportion of the different sugars incidental to the ripening, and this method of investigation was subsequently extended by Ricciardi² and by Gerber³ whose data are here summarized in tabular form in order to show the comparative carbohydrate content of banana pulp at different stages of ripeness.

TABLE I.

CARBOHYDRATE CONTENT OF BANANA PULP AT DIFFERENT STAGES OF RIPENESS.

Color of Peel (Evidence of Ripeness).	Reducing Sugars Cal- culated as Glucose (Invert Sugar).	Total Sugars Cal- culated as Glucose (Invert Sugar + Cane Sugar).	Total Carbohydrate (Starch, Sugars, etc).
	Per Cent.	Per Cent.	Per Cent.
Green.....	1.24	7.76	21.51
Green-yellow.....	1.24	7.76	21.51
Yellow.....	6.43	17.48	22.12
Yellow-brown.....	8.55	19.00	20.00
Yellow-brown.....	11.05	18.26	19.00
Brown.....	10.90	16.60	16.60

The conspicuous change in the carbohydrates consists in a gradual transformation of starch into soluble carbohydrates accompanied by a marked decrease in the total carbohydrate content of the pulp. The following table combining König's compilation⁴ and recent analyses published by Chace, Tolman, and Munson⁵ serves to give an idea of the range in composition of the ripe pulp of different varieties.

¹ Corenwinder, *Comptes Rendus*, lvii, p. 781, 1863; lxxxviii, p. 293, 1879

² Ricciardi, *Comptes Rendus*, lxxxv, p. 393, 1882.

³ Gerber, *Annales des sciences naturelles*, Series VIII, p. 4, 1896.

⁴ König, *Chemie der menschlichen Nahrungs- und Genussmittel*, i, pp 851-2, 1903.

⁵ Chace, Tolman, and Munson, United States Department of Agriculture, Bureau of Chemistry, Bulletin No. 87, 1904.

TABLE II.

COMPOSITION OF DIFFERENT VARIETIES OF RIPE BANANAS.

Variety.	Solids. Per Cent.	Protein. Per Cent.	Fat. Per Cent.	Sugar.			Other nitrogen- free extract.	Fiber. Per Cent.	Ash. Per Cent.
				Invert. Per Cent.	Cane. Per Cent.	Total. Per Cent.			
From Brazil (a)	27.60	2.14	0.96	—	—	14.40	8.60	0.38	1.03
Venezuela (b)	26.20	1.60	0.30	—	—	12.30	10.70	0.20	1.10
Sandwich Islands (c)	27.94	0.61	0.30	—	—	16.25	—	—	1.08
America (d)	25.90	1.20	0.80	—	—	22.90	—	—	1.00
Unidentified (e)	27.60	1.44	0.09	—	—	21.90	2.03	1.22	0.92
Cuba, Nino (f)	28.09	—	—	20.42	0.19	20.61	—	—	0.70
Manzano (f)	30.92	—	—	19.66	0.30	19.96	—	—	0.70
Manzano (f)	30.55	—	—	—	—	—	—	—	0.85
Unidentified (f)	30.44	1.36	—	21.43	0.30	21.73	—	—	0.77
Indiano (f)	27.06	0.89	—	8.44	13.17	21.60	—	—	0.98
Indiano (f)	27.16	0.84	—	17.06	—	17.06	—	—	1.10
Johnson (f)	24.34	1.13	—	14.60	5.20	19.80	—	—	0.82
Johnson (f)	26.13	1.13	—	7.88	13.83	21.71	—	—	0.85
Johnson (f)	26.24	1.21	—	8.49	13.27	21.76	—	—	0.86
Ciento a la boca (f)	31.97	1.23	—	8.07	17.59	25.66	—	—	0.82
Ciento a la boca (f)	34.55	1.22	—	7.35	—	—	—	—	0.93
Colorado (red) (f)	21.60	1.18	—	5.78	11.35	17.13	—	—	0.83
Colorado (red) (f)	25.16	1.21	—	9.56	10.36	19.92	—	—	0.86
Oronoco (red) (f)	23.34	1.33	—	3.16	12.20	15.36	—	—	0.80
Oronoco (red) (f)	25.51	—	—	—	16.54	—	—	—	1.08

(a) Corenwinder, 1876.

(b) Marciano and Muntz, 1879.

(c) G. E. Colby, 1893.

(d) Atwater and Bryant, 1895

(e) Balland, 1900.

(f) Chace, Tolman, and Munson, 1904.

The distinct amylolysis which goes on side by side with the loss of carbohydrate in the ripening pulp suggested a search for enzymes of the amylase (ptyalin) type in both the green and ripe fruit. The negative results which followed the attempts to isolate such a soluble ferment in the laboratory led Professor Mendel to advise me to make a more careful investigation of the conditions under which the ripening transformation proceeds, as a preliminary step to the study of the metabolic changes involved. It is apparent from the figures already given that the extent of ripening attained in the fruit at any given time is evidenced approximately by the ratio of soluble to insoluble carbohydrate. These factors were therefore determined by the following methods.

METHODS.

The peel of the fruit was removed and the pulp cut into thin slices.

After passing it through a meat chopper two 25-gram portions were weighed out, one for the determination of total carbohydrate, and the other for soluble carbohydrate.

Total carbohydrate: The material, further macerated in a mortar if necessary, was introduced into a 500 c.c. volumetric flask with 200 c.c. of water; 20 c.c. of 25 per cent. hydrochloric acid were added and the mixture digested in a water bath at boiling temperature for three hours. The solution was then nearly neutralized with sodium hydroxide solution, 5 c.c. of basic lead acetate added, the solution made up to the mark and filtered. Of the filtrate 50 c.c. were taken in a 100 c.c. flask, 1 c.c. of 60 per cent. sulphuric acid added to remove the lead, the solution made up to the mark and again filtered. Of this filtrate 25 c.c. were used for the reduction of Fehling's solution. The results are expressed in terms of dextrose.

Soluble carbohydrate: The material, further macerated if necessary, was introduced into a 500 c.c. volumetric flask with 300 c.c. of lukewarm water and allowed to stand with frequent shaking for at least five hours. The solution was then made up to the mark and filtered. Of the filtrate 50 c.c. were taken in a 100 c.c. flask, 5 c.c. of concentrated hydrochloric acid added, and heated at 70° C. for ten minutes. The solution was then cooled, nearly neutralized, made up to the mark and filtered if necessary. (A slight flocky precipitate sometimes appeared at this point.) Of this filtrate 25 c.c. were used for the reduction of Fehling's solution. The results are expressed in terms of dextrose as above.

Insoluble carbohydrate was obtained by difference except in case of autolysis trials when it was determined directly in the thoroughly extracted and washed residue by the method already described for the determination of total carbohydrate.

Two analyses of unripe banana pulp, made to test the accuracy of this method of procedure, are given below.

TABLE III.

TEST ANALYSES OF UNRIPE BANANA PULP.

Series & No.	Carbohydrate (as dextrose).			
	Soluble. Per Cent.	Insoluble (calculated). Per Cent.	Insoluble (determined). Per Cent.	Total Per Cent.
I, 5	1.37	24.83	24.08	26.20
X, 7	1.07	24.13	23.84	25.20

Entirely green bananas were always selected from the same bunch, care being taken to have them as nearly perfect and uniform in size as possible. The exteriors were thoroughly scrubbed with a brush, the cut ends seared with a hot rod and sealed with paraffin. For control observations one fruit treated

n this manner was analyzed at once, while others were allowed to ripen normally in the air and examined when desired.

Results are here given showing the relative amounts of carbohydrates in pulps of bananas which have been placed under modified conditions of gaseous exchange. The specimens in each series are comparable in having been taken at the same time from one bunch.

TABLE IV.

CARBOHYDRATE CONTENT OF BANANAS UNDER MODIFIED CONDITIONS OF GASEOUS EXCHANGE.

Series & No.	Carbohydrate (as dextrose).			Remarks.
	Soluble. Per Cent.	Insoluble. Per Cent.	Total. Per Cent.	
I, 4	1.30	25.26	26.56	Control analysis of unripe pulp.
1	17.02	1.98	19.00	Control analysis of ripe pulp.
2	1.65	22.19	23.84	Green banana coated with paraffin (twenty-one days).
VI, 12	2.68	21.40	24.08	Control analysis of unripe pulp.
4	16.86	3.39	20.25	Control analysis of ripe pulp (six days).
2	17.40	4.44	21.84	Banana imperfectly coated with paraffin (six days).
5	—	—	19.00	Banana ripened six days—then coated with paraffin (six days).
20b	14.72	3.36	18.08	Banana coated with a gum mastic varnish (nineteen days).
II, 10	1.21	27.03	28.24	Control analysis of unripe pulp.
2a	18.80	—	—	Control analysis of ripe pulp.
5	0.95	24.17	25.12	Banana submerged in oil (fourteen days).

The preceding typical experiments indicate clearly that the ripening (formation of soluble carbohydrates and decrease of total carbohydrates) proceeds slowly, if at all, when the gaseous exchange is prevented by some inert coating. This is seen especially in the failure to produce soluble carbohydrates; moreover the characteristic color-changes incidental to normal ripening were inhibited. An indirect explanation of this inhibition was suggested by a further study of the carbohydrate changes in bananas maintained in various gases and in vacuo at room temperature, as indicated in the table below. The data from the same series are directly comparable.

TABLE V.

CARBOHYDRATE CONTENT OF BANANAS SUBJECTED TO ABNORMAL ATMOSPHERES.

Series & No.	Carbohydrate (as dextrose).			Remarks.
	Soluble. Per Cent.	Insoluble. Per Cent.	Total. Per Cent.	
II, 10	1.21	27.03	28.24	Control analysis of unripe pulp.
2a	18.80	—	—	Control analysis of ripe pulp.
6	1.02	25.14	26.16	Banana in atmosphere of carbon dioxid (twenty-one days).
VI, 12	2.68	21.40	24.08	Control analysis of unripe pulp.
4	16.86	3.39	20.25	Control analysis of ripe pulp (six days).
13	12.81	9.59	22.40	Control analysis of ripe pulp (four days).
9	13.45	8.39	21.84	Banana in atmosphere of oxygen (four days).
19S	—	—	20.00	Banana in air (five days).
15S	—	—	24.72	Banana in atmosphere of carbon dioxid (five days).
17S	7.96	9.32	17.28	Banana in atmosphere of hydrogen (thirty-five days).
14S	5.12	15.84	20.96	Banana in atmosphere of illuminating gas (thirty-five days).
3	4.86	16.90	21.76	Banana in a vacuum (forty-two days).
IX, 9	2.26	23.42	25.68	Control analysis of unripe pulp.
8	16.27	5.09	21.36	Control analysis of ripe pulp (six days).
2	4.44	18.56	23.00	Banana in a frequently changed atmosphere of carbon dioxid (six days).
3	13.11	8.81	21.92	Banana in a closed air-chamber: air frequently changed.
1	16.84	3.16	20.00	Banana in frequently changed atmosphere of oxygen (six days).

The inhibitory action of a deficiency or a complete lack of oxygen is plainly indicated by these analyses. Physical changes were manifested which agreed with the observations of early investigators, notably Lechartier and Bellamy.¹ The skin became brown and later decidedly black, and the cells were no longer active. The pulp also underwent a pronounced change in texture and color. The accumulated respiratory products themselves seem to be inhibitory to the ripening processes (cf. IX, 3). The significance of these physiological alterations will be studied later.

¹ Lechartier and Bellamy: Referred to by E. Duclaux, *Traité de microbiologie*, iii, p. 41 et seq., 1900.

An attempt was made to determine the agents by which the carbohydrate conversions are carried out during the ripening of the banana. Amylases have not yet been detected in toluol-water extracts of the macerated unripe pulp, of the unripe pulp and scrapings of the inner surface of the peel, or of the partially ripened pulp, in tests with arrowroot paste for twenty-four hours at 38° C. Autolysis trials with the pulp of both green and ripening bananas, carried out in the presence of water and toluol, have failed to give evidence of any enzymatic changes. The character of some of the experiments is indicated in the following table.

TABLE VI.

CARBOHYDRATE CONTENT OF BANANAS SUBJECTED TO AUTOLYSIS.

Series & No.	Carbohydrate (as dextrose).			Remarks.
	Soluble. Per Cent.	Insoluble. Per Cent	Total. Per Cent	
I, 4	1.30	25.26	26.56	Control analysis of unripe pulp.
1	17.02	1.98	19.00	Control analysis of ripe pulp.
3a	1.57	24.32	25.89	Macerated banana pulp autolyzed at 15° C. (fourteen days).
3b	1.42	24.72	25.44	Macerated banana pulp autolyzed at 15° C. (twenty-one days).
II, 9a	1.21	21.52	22.73	Control analysis of unripe pulp and inner layers of peel.
9b	1.58	22.00	23.58	Unripe banana pulp and inner layers of peel autolyzed at 15° C. (seven days).
2a	18.80	—	—	Control analysis of ripe pulp (seven days).
2b	19.10	—	—	Analysis of a portion of 2a autolyzed seven days after having ripened seven days.
X, 7	1.07	23.84	24.91	Control analysis of unripe pulp.
1	1.79	22.16	23.95	Banana pulp autolyzed at 15° C. (ten days).
3	2.13	24.64	26.77	Banana pulp autolyzed at 38° C. (ten days).
4	1.92	21.12	23.14	Banana pulp autolyzed at 15° C. with air bubbling through (ten days).

The study of the problems involved is being continued in various directions in this laboratory.

EXTREME TOXICITY OF SODIUM CHLORIDE AND ITS PREVENTION BY OTHER SALTS.

By W. J. V. OSTERHOUT.

(Received for publication, December 22, 1905.)

A pure sodium chloride solution, at the concentration in which this salt exists in sea-water, may be poisonous to marine organisms, as was first proven by Loeb,¹ who found that the young of a marine fish (*Fundulus*) soon die in $\frac{5M}{8}$ to $\frac{M}{3}$ solution of sodium chloride.

That death is due to the poisonous action of the sodium chloride, and not merely to the absence of other constituents of the sea-water, was shown by control experiments with distilled water, in which the young fish lived indefinitely. It would, as Loeb remarks, be impossible to prove the toxicity of sodium chloride were not the organism capable of living in distilled water.

In botanical literature, there are recorded very few experiments on the toxicity of sodium chloride, where control experiments were made with distilled water. The germination of seeds, which takes place readily in distilled water, is said to be inhibited by solutions of sodium chloride ranging from two to eleven per cent., and solutions as weak as five-tenths per cent. may exert a slightly injurious influence.² Lindforss³ states that pollen grains of some plants will not grow in one-tenth per cent. sodium chloride although they do so in distilled water.

It is stated by True⁴ and Klebs⁵ that $\frac{M}{10}$ sodium chloride

¹ *Amer. Journ. Physiol.*, iii, p. 327, 1900.

² Zeller, Inaugural Dissertation, 1826; Fleischer, see Nobbe, *Samenkunde*, p. 269, 1876; Tautphöus, *Centralblatt f. Agriculturchemie*, ix, p. 351; Nessler, *Centralblatt f. Agriculturchemie*, xii, p. 125; Jarius, *Landw. Versuchsstation*, xxxii, p. 162, 1886; Coupin, *Revue gen. de botan.*, x, p. 177, 1898.

³ *Pringsheim's Jahrb.*, xxix, p. 36, 1896.

⁴ *Botan. Gaz.*, xxvi, p. 407, 1898.

⁵ Klebs, *Bedingungen der Fortpflanzung bei einigen Algen und Pilzen*, p. 548, 1896; see Pfeffer, *Pflanzenphysiologie*, 2^{te} Aufl., ii, p. 330, 1904.

solutions are less favorable for the growth and reproduction of algæ than isotonic solutions of cane sugar.

In all these botanical experiments the sodium chloride solutions employed were very much stronger (one hundred to one thousand times) than those to which plants are exposed in nature. These experiments indicate therefore only a weak degree of toxicity.

I have been unable to find any statement that sodium chloride is injurious in dilute solutions. The prevailing view is undoubtedly that expressed by Pfeffer¹: "Speciell die Lithiumsalze üben auf höhere und niedere Pflanzen schon bei ziemlicher Verdünnung eine schädigende Wirkung aus. Eine solche kommt aber in keiner Weise den Salzen des Na zu."

It will be shown in this paper that sodium chloride may exert a markedly poisonous action at great dilutions.

The best material which I have found for demonstrating this action is *Vaucheria sessilis*, a green alga common upon moist earth and in running water. Small tufts of the material, washed free from dirt, were placed in glass dishes and covered with water. Glass slides were placed upright in these dishes, dipping half an inch into the water. On the following morning numerous zoöspores were found to have attached themselves to the slides along the water line, from fifty to one hundred being found closely clustered together in a narrow band extending the width of the slide, thus making subsequent observation and counting an easy matter. The zoöspores were large enough to be visible to the naked eye and could be easily observed with a hand lens without removing them from the solutions. They could also be removed for a short time and observed under the microscope without injuring them.

The slides, with the attached zoöspores, were rinsed for three or four hours in several changes of distilled water and then transferred to glass tumblers, each containing one hundred cubic centimeters of solution. These were covered with glass plates, to exclude dust and hinder evaporation. The zoöspores were in all cases well submerged in the solution.

Material from certain localities uniformly gives zoöspores which

¹*Pflanzenphysiologie*, 2^{te} Aufl., i, p. 423, 1897.

soon die in distilled water,¹ while that from certain other localities yields zoöspores which live three or four weeks or even longer in distilled water.² Zoöspores of the latter sort were used exclusively in these experiments.

Such zoöspores, if thoroughly rinsed in several changes of distilled water (three or four hours) and placed in sodium chloride $\frac{3M}{2}$, usually die within a few minutes. Young plants (five to ten times as long as the zoöspores) are even more sensitive and show the beginning of disorganization of the protoplasm within four or five minutes. This appearance of the protoplasm permits an accurate determination of the death point.

This poisonous effect is easily observable in dilute solutions. Young plants placed in $\frac{1M}{100}$ sodium chloride are completely killed in ten to fifteen hours; in $\frac{1M}{400}$ sodium chloride, one-half succumb in three and one-half to four days, the remainder dying off irregularly. In $\frac{1M}{2500}$ sodium chloride, one-half are dead in eight to ten days or even sooner. In $\frac{1M}{10000}$, a large proportion, usually more than one-half, are dead in twelve days, the rest dying off irregularly. The controls in distilled water not only remain normal in appearance but continue to grow for three or four weeks.

In order to make certain that this extreme toxicity was not due to any impurity in the sodium chloride the experiments were repeated with the purest salts obtainable from the best manufacturers. The result was the same. A quantity of Kahlbaum's chemically pure sodium chloride was then recrystallized six times from solutions in distilled water. The salt so obtained was freed from the last trace of potassium by heating to redness in a platinum crucible until it gave no flame test for potassium. The residue was then dissolved in distilled water and its concentration determined by titration. This pure salt gave the same results as before.

In order to guard against possible acidity, control experiments

¹ Special precautions were taken in regard to the water, which was distilled from glass only, since traces of heavy metals, especially copper, are highly poisonous. The first third of the distillate was rejected to avoid contamination by ammonia.

² This may indicate the existence of physiological varieties. To judge from the literature, distilled water is generally regarded as non-poisonous for plants.

were made in which the solutions were rendered faintly alkaline by sodium bicarbonate. This, however, did not change the results.

It appears certain that for these plants sodium chloride is poisonous even in $\frac{1}{100000}$ solution.

A determination of the amount of sodium in the running water of the brook in which the plants grew was kindly made by Professor Geo. E. Colby, Assistant Professor of Agricultural Chemistry. The sodium content amounts to about $\frac{1}{100000}$. Evidently the poisonous action which sodium would exert at this concentration is inhibited by other constituents of the water. Which constituents act as an antidote to the sodium?

Before attempting to answer this question I determined the maximal concentration in which the algæ will grow, since it is more convenient to work with stronger solutions. In sea-water containing six-tenths per cent. total salts the algæ grow excellently. Sea-water of this concentration is approximately imitated by the following mixture:

$\frac{3}{32}$ NaCl	1000 c.c.
" MgCl ₂	78 "
" MgSO ₄	38 "
" KCl	22 "
" CaCl ₂	10 "

In this mixture the plants developed in normal fashion and produced mature fruit.

Young plants placed in $\frac{3}{32}$ sodium chloride live only a few minutes; on adding magnesium chloride, magnesium sulphate, or potassium chloride in the proportions given above, only a little improvement was produced (less by potassium chloride than by magnesium chloride or magnesium sulphate). But on adding ten cubic centimeters of $\frac{3}{32}$ calcium chloride to one thousand cubic centimeters of $\frac{3}{32}$ sodium chloride, the poisonous effect of the sodium chloride totally disappeared, so that the plants lived as long as in distilled water (two to four weeks). The further addition of potassium chloride to the mixture enables the plants to live six to eight weeks; still further addition of magnesium chloride and sulphate (thus completing the mixture) produces a medium in which they live and grow indefinitely, thriving even better than in tap-water and producing normal mature fruit. Sodium chloride plus magnesium chloride plus

calcium chloride is even better than sodium chloride plus potassium chloride plus calcium chloride.

The average behavior of the plants in the various solutions is shown in the following table:

Solution.	Length reached in 35 days	Development reached in 35 days.	Duration of life.
Dilute artificial sea-water.			
3/32 M NaCl 1000 c.c.	16.6 mm.	Normal appearance Rhizoids Mature fruit	Lives indefinitely
" MgCl ₂ 78 "			
" MgSO ₄ 38 "			
" KCl 22 "			
" CaCl ₂ 10 "			
Dilute natural sea-water Total salts = 0.6 per cent.	12 mm.	Normal appearance Rhizoids Mature fruit	Lives indefinitely
Tap-water, containing .0015 M Na .000095 M K .00117 M Mg .00102 M Ca	7 mm.	Normal appearance Rhizoids No fruit	Lives indefinitely
3/32 M NaCl 1000 c.c.	4.6 mm.	Normal appearance Rhizoids No fruit	6 to 8 weeks
" KCl 22 "			
" CaCl ₂ 10 "			
3/32 M NaCl 1000 c.c.	2 2 mm.	Normal appearance No rhizoids No fruit	2 to 4 weeks
" CaCl ₂ 10 "			
3/32 M NaCl		No growth	10 to 20 minutes
$\frac{M}{147}$ MgCl ₂ = { dist. water 1000 c.c. 3/32 M MgCl ₂ 78 "		"	1 to 1 days
$\frac{M}{151}$ MgSO ₄ = { dist. water 1000 c.c. 3/32 M MgSO ₄ 38 "		"	1½ to 2½ days
$\frac{M}{155}$ KCl = { dist. water 1000 c.c. 3/32 M KCl 22 "		"	2 to 3 days
$\frac{M}{167}$ CaCl ₂ = { dist. water 1000 c.c. 3/32 M CaCl ₂ 10 "		"	8 to 10 days
3/32 M NaCl 1000 c.c.			12 to 18 hours
" MgCl ₂ 78 "			
3/32 M NaCl 1000 c.c.			2 to 4 hours
" KCl 22 "			
3/32 M NaCl 1000 c.c.			12 to 20 hours
" MgCl ₂ 78 "			
" KCl 22 "			
3/32 M NaCl 1000 c.c.	14 mm.	Rather pale appearance Rhizoids Immature fruit	8 to 10 weeks
" MgCl ₂ 78 "			
" CaCl ₂ 10 "			
Distilled water	2.2 mm	Normal appearance No rhizoids No fruit	3 to 4 weeks

Inasmuch as algæ are very sensitive to acids, parallel series of experiments were made in which all solutions were rendered faintly alkaline by sodium bicarbonate. Some slight differences were observable at first, but at the end of thirty-five days these had completely disappeared.

It may be remarked that for these plants potassium chloride, magnesium chloride, and magnesium sulphate are nearly as toxic as sodium chloride, while calcium chloride is not far behind in this respect. We have here a striking illustration of the remarkable fact that when salts, individually poisonous, are mixed together in the right proportions their toxicity totally disappears.¹ Such a mixture has been termed a balanced solution by Loeb, who has emphasized the fact that the blood as well as the sea-water is in fact such a solution. These results from the study of plants are in striking accord with those obtained by Loeb² on marine animals as well as with those obtained by Ostwald³ on fresh-water animals. The biological principle here involved is evidently of very general application.

These experiments were carried on in the Rudolph Spreckels Physiological Laboratory, the facilities of which were generously placed at my disposal by Professor Loeb, for whose cordial co-operation I desire to express my sincere thanks.

RESULTS.

1. Young plants of a fresh-water alga, *Vaucheria sessilis*, which can live three to four weeks in distilled water, are killed in a few minutes by $\frac{3M}{33}$ sodium chloride and in a few days by $\frac{M}{10000}$ sodium chloride.

2. The toxicity of a $\frac{3M}{33}$ sodium chloride solution is inhibited by addition of calcium chloride in the proportion of one hundred parts of sodium chloride to one of calcium chloride.

¹ That we are here dealing with effects of kations is shown by two things: (1) the dilutions involved, and (2) the disappearance of toxicity without appreciably altering the concentration of chlorine ions.

² *Amer. Journ. Physiol.*, iii, p. 327, 1900; *Pflüger's Arch. f. d. ges. Physiol.*, xcvii, p. 394, 1903; cvii, p. 252, 1905; *University of California Publications, Physiology*, i, p. 55, 1903.

³ *Pflüger's Arch. f. d. ges. Physiol.*, cvi, p. 568, 1905; *University of California Publications, Physiology*, ii, p. 163, 1905.

3. The plants are able to develop normally and produce mature fruit in a $\frac{3}{4}$ M sodium chloride solution to which have been added small quantities of magnesium chloride, magnesium sulphate, potassium chloride, and calcium chloride, all of which salts are toxic when used singly.

Note.—The poisonous effects of the very dilute solutions of sodium chloride mentioned on page 365 were uniformly obtained with material from a particular locality. Material from other localities proved less sensitive to the action of sodium chloride. Before germination, the zoöspores are less sensitive than shortly after it.



EXPERIMENTS TO DETERMINE THE INFLUENCE OF THE BROMIDS OF BARIUM AND RADIUM ON PROTEIN METABOLISM.¹

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¹ This investigation was one of a series of studies begun in this labor-
atory about a year ago. A preliminary communication of its results was
made last May. See Gies and collaborators: *Proc. Soc. for Exper. Biol. and
Med.*, ii, p. 86, 1905; also, *Science*, xxi, p. 986, 1905; *Amer. Med.*, ix, p.
1026, 1905; and *Medical News*, xlii, p. 520, 1905.

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I.—INTRODUCTION.

Interest in the properties and significance of radium has increased among biologists with each new discovery bearing on the physical nature of that remarkable element. The widespread desire of medical men to make use of whatever therapeutic effects radio-active materials may exercise has led to many experiments on the *biophysical* influence of radium; with results, however, that are far from being definite and concordant. Thus far, very little information regarding the *biochemical* effects of radium has been accumulated. It was the special purpose of this study to determine, if possible, the general metabolic influence of small doses of radium bromid. So far as we are aware, no previous investigation of this nature has been made with radium.

It was our intention, in this research, to study the effects of radium bromid administered per os and also subcutaneously. The prohibitive cost of radium salts made the use of more than very slight quantities impossible, and the investigation could not have been carried out at all had it not been for the generosity of Mr. Hugo Lieber, who freely gave us a relatively large supply of expensive material for the purpose. Three different preparations of radium bromid were given to us by Mr. Lieber. They manifested activities of 240, 1,000, and 10,000 respectively. Assuming the activity of pure radium to be 1,000,000,¹ and the activity of any radium preparation to be roughly proportional

¹ "No definite results have as yet been given on the activity of pure radium (*radium chlorid* ?), but the Curies estimate that it is about 1,000,000 times that of uranium and may possibly be still higher. The difficulty of making a numerical estimate for such an intensely active body is very great."—Rutherford: *Radio-activity*, p. 14, 2d ed., 1905; also p. 14, 1st ed., 1904.

to the amount of radium present (this of course, is only approximately true), it follows that the amount of radium bromid actually present in the preparations used was very small. On this basis, the radium preparation of 240 activity contained 0.024 per cent. of radium bromid; the preparation of 1,000 activity contained 0.1 per cent., and the preparation of 10,000 activity contained 1.0 per cent. These preparations of relatively low activity were composed chiefly of barium bromid.¹ Therefore control experiments were carefully conducted to ascertain the metabolic effects of barium bromid, and to enable us to be certain (if not by direct means, at least by difference) of the effects of the radium in the radio-active preparations employed. This course with respect to barium appeared to be all the more desirable because of the fact that no previous study of its general metabolic influence seems to have been made.²

II.—DESCRIPTION OF THE EXPERIMENTS.

Preliminary Experiments on the Toxicity of Barium Bromid.

Eight preliminary experiments to determine the toxicity of barium bromid were made, in order to ascertain the largest

¹ They were made by the Curie method. "Radium is extracted from pitchblende by the process used to separate barium, to which radium is very closely allied in chemical properties. After the removal of other substances, the radium remains behind mixed with barium. It can, however, be partially separated from the latter by the difference in solubility of the chlorids in water, alcohol, or hydrochloric acid. The chlorid of radium is less soluble than that of barium, and can be separated from it by the method of fractional crystallization. After a large number of precipitations, the radium can be freed almost completely from the barium." —Rutherford, *loc. cit.*, p. 13.

² Among the more important papers on the biological effects of barium are the following: Brodie, *Philosoph. Trans. Royal Soc.*, London, cii, p. 219, 1812; Orfila, *Toxicologie*, i, p. 194, 1829, Paris; Orfila, *Traité de Toxicologie*, i, p. 309, 2d ed., 1852, Paris; Onsum, *Virchow's Arch. f. path. Anat.*, etc., xxviii, p. 233, 1863; Cyon, *Reichert's Arch. f. Anat., Physiol. u. wissenschaft. Med.*, p. 196, 1866; Mickwitz, *Vergleich. Untersuch. d. physiol. Wirk. d. Salze. d. Alkalien. u. alkalischer Erden*. Dissert., Dorpat, 1874; Brunton and Cash, *Proceed. Royal Soc.*, London, xxxv, p. 63, 1883; Neumann, *Pflüger's Arch. f. d. ges. Physiol.*, xxxvi, p. 576, 1885; Bary, *Beiträge z. Baryumwirkung.*, Dissert., Dorpat, 1888; Sommer, *Beiträge z. Kennt. d. Baryumvergift.*, Dissert., Würzburg, 1890; MacCallum *Amer. Jour. Physiol.*, x, pp. 109, 259, 1903-1904.

amount which could be given to dogs without causing serious metabolic disturbances. The dogs used in these experiments were all common, healthy mongrels. To a large extent, the results obtained in the preliminary observations determined the amounts of barium bromid used in the control metabolism experiments.

Administration per os. Experiments I-V.

Experiment I.—A dog weighing 22.8 kilos was fed on an approximately normal diet consisting of hashed meat, cracker-meal, lard, bone-ash, and water. Barium bromid was administered in small balls of the meat which were swallowed without mastication.

The amount of barium bromid given on the first day was 1 mg. per kilo. The daily dose was increased 1 mg. per kilo until the 11th day, when it amounted to 11 mg. per kilo. During the following period of eight days ending with the 19th day of the experiment, the daily increase in the dose was approximately 5 mg. per kilo. The actual dose on the 19th day was 50 mg. per kilo. This was followed by a five-day period in which the daily increase in the dose was 10 mg. per kilo. No effects were noticed up to the end of the 21st day when a total of 10.3954 grams had been given; but on the last three days of this period, ending with the 24th day, vomiting, salivation, and slight diarrhoea occurred. The desire for food was also diminished. On the 24th day, the last of this period, the dog received 99 mg. per kilo. or 2.2628 grams, which made the total dose up to this point 16.4988 grams.

The amount of administered barium bromid was then raised to 213 mg. per kilo without even causing a recurrence of the symptoms noticed on the preceding days. The total dose at this time was 21.3500 grams. With a dose of 266 mg. per kilo. on the following day, however, the symptoms reappeared. On the 26th day, the dog was fed 300 mg. per kilo. General diminution of appetite was observed. On the 27th day, the dog was very thirsty, but ate only a small portion of the regular diet. No barium bromid was given on this day. Shortly after feeding, the dog eagerly drank more water,

Within the six hours immediately after feeding, vomiting and defecation took place, and at the end of that time, the animal seemed to be greatly distressed. Salivation also occurred about this time. These symptoms became more and more marked, the breathing became labored and rapid, and there was rise of temperature. Paralysis was noticed especially in the hind legs. Then followed general prostration; head movements accompanying respiratory efforts became very decided and there was also marked twitching of the abdominal muscles. The animal rapidly became unconscious. Heart-beat at this stage was about 142 per minute. The symptoms increased in intensity until death ensued about three hours after the first marked symptoms appeared. This was thirty-six hours after the last dose of barium bromid had been administered. Death seemed

to have resulted from asphyxia. There were no terminal convulsions.

Autopsy showed that nearly all the parts were normal. There was an area of induration, about three-fourths of an inch in diameter, at the fundus of the stomach. This was elevated, reddened, and smooth on the inside, but did not extend to the outside. The intestines were somewhat similarly affected.

The stomach contents, about 150 c.c. in volume, were acid in reaction and on further acidification with hydrochloric acid formed no precipitate with dilute sulfuric acid.

Experiment II.—A small dog weighing 11.1 kilos was fed on a diet similar to that described in Experiment I. The administered amounts of barium bromid per kilo were exactly the same daily up to 11 mg. per kilo—the dose on the 11th day. Then the dose was increased 6 mg. per kilo, making it 17 mg. per kilo for the 12th day. During the next period of seven days ending with the 19th day of the experiment, the daily increase in the dose was 5 mg. per kilo. The dose for the 19th day was 52 mg. per kilo. This was followed by a five-day period during which the daily increase in the dose was 10 mg. per kilo. No effects were noticed up to the end of the 21st day when a total of 5.2836 grams had been given. On the last three days of this period which ended with the 24th day, the symptoms were similar in all respects to those described for corresponding days in Experiment I. This is also true of the 25th day, on which the dose was raised to 204 mg. per kilo; and of the 26th day with a dose of 263 mg. per kilo. The total dose administered was then 12 4401 grams. On the 27th day, the only barium bromid administered was that still contained in the ball of meat of the previous day's feeding, which the dog had vomited. This ball was vomited a few minutes after feeding but eaten again. Vomiting then occurred at short intervals for one and one-half hours, when general prostration became apparent. Loss of power in the hind legs was first noticed, then in the fore legs. Labored breathing and increase in the rapidity of the heart-beat were observed. The following day, the dog was in a state of complete prostration. Water was refused and long-continued gasps for breath became very marked. Death ensued twenty-five hours after the feeding of the meat-ball on the 27th day.

Autopsy showed the intestines colored bluish, due to the venous congestion. The left ventricle was full of black blood. In the fundus of the stomach was found a circular spot of induration about one inch in diameter, which was colored a darker red than the rest of the membrane. The thickness of the stomach wall was markedly increased at this spot. The duodenum showed areas of inflammation. Otherwise no abnormality was observed.

Experiment III.—A small dog weighing 5 kilos was fed on a diet similar to those of the preceding experiments. In addition, a dose of barium bromid equal to 25 mg. per kilo was administered. About three and one-half hours later, a second dose of 100 mg. per kilo was given. This made

a total of 0.6250 grams or 125 mg. per kilo. An hour after the feeding of the larger dose, the dog seemed less active and also feverish, but in two and one-half hours was apparently normal again. Another dose of 200 mg. per kilo was then given. This made a total of 1.625 grams or 325 mg. per kilo. During the next three and one-half hours vomiting occurred, but the dog did not appear to be particularly ill. One of the balls of meat containing barium bromid was vomited. Diarrhœa occurred during the night. On the following morning, the animal was helpless. He was extremely thirsty, but refused all food. On the following day, the dog had apparently completely recovered. Two days later, diarrhœa was noticed but no further symptoms appeared.

Experiment IV.—A dog weighing 8.37 kilos was fed as usual except that bone-ash was omitted from the diet. A dose of 200 mg. per kilo of the barium bromid was fed. Vomiting and salivation were the only symptoms that appeared during the following eight and one-half hours, and these symptoms were not very marked. For two days following this dose, the dog was apparently normal, and observation was discontinued.

Experiment V.—In this experiment a dog weighing 10.40 kilos was fed a diet of the customary quantities. Bone-ash was excluded. 100 mg. per kilo of barium bromid were given. Inactivity was the only symptom that appeared. On the following day, the dog seemed normal. No vomiting or diarrhœa occurred. On the third day, also, the animal was apparently quite normal and observation was discontinued.

Subcutaneous Injection—Experiments VI-VIII.

Experiment VI.—A dog weighing 18.25 kilos, fed as described in the first experiment, received subcutaneously 5 mg. per kilo of barium bromid in 2 c.c.¹ of aqueous solution. Within five minutes, respiration was increased and a feverish condition soon followed. The hind legs of the dog became affected half an hour after the injection. Salivation appeared in forty-five minutes. In an hour and a half, tremor appeared and the heart became irregular. Vomiting also occurred. The pulse increased in rapidity until it reached 128 per minute seven hours after the injection, at which time it was extremely irregular. About this time, the dog drank water eagerly, and two hours later was very much improved.

On the following day, the animal was apparently normal. The appetite was not affected. Sometime after feeding, vomiting occurred; but all of the vomited material was eaten again. The animal seemed tired.

On the third morning, it was observed that vomiting had occurred to a slight extent during the night. The dog's movements were sluggish. Only a portion of the regular diet was eaten: and later there occurred attempts to vomit, but only a little fluid was brought up.

On the fourth day, the dog was apparently normal and ate eagerly

¹ In the subsequent experiments the volumes of the solutions never exceeded the above quantity.

all of a meal equal in quantity to that fed at the beginning of the experiment. At this point the experiment was discontinued.

Experiment VII.—A dog weighing 3.43 kilos was fed as described in Experiment I. The intention was to inject subcutaneously 2.5 mg. per kilo. During the injection there was an accidental loss of about one-fourth of the solution. Three and one-half hours after the injection no marked symptoms appeared.

On account of our uncertainty as to the actual quantity of barium bromid injected, the experiment was repeated later on a new dog. The animal that had received the incomplete dose was continued, however, on the usual diet and kept under observation. Seven days later (during which period no marked symptoms were apparent) the hind legs of the dog were greatly weakened. Salivation became marked. On the 9th day, the dog was completely prostrated and death occurred at 12.45 P. M. or about one hundred and ninety-three hours after the injection of the barium bromid.

Autopsy revealed nothing abnormal excepting the lungs and the pleural cavity. The latter had on the left side, especially, a serofibrinous exudate, which could be easily pulled from the chest wall and the lung. The surface of the left lung was studded with a few hard, somewhat grayish patches, which broke on the application of slight pressure, exuding a fluid. The left lung was red, while the right lung was almost white and was irregularly studded with red areas especially in the middle lobe. Both lungs had a mottled appearance. No trace of inflammation was found under the skin at the point of injection.

Experiment VIII.—In this experiment, a dog weighing 18.06 kilos was used. The animal was fed on a diet similar to that described in Experiment I. Barium bromid, in quantity equal to 25 mg. per kilo, was injected subcutaneously. Twenty minutes after the injection slight salivation was noticed and shortly afterwards vomiting occurred. Four minutes after the first signs of salivation appeared, the saliva ran in streams from the dog's mouth. A rise in temperature was also observed. During the following ten minutes, weakening of the legs occurred, but salivation seemed to have abated somewhat. During the next six hours, the animal was considerably depressed, and the symptoms, salivation, feverishness, labored breathing, irregular and fluttering heart-beat, and restlessness, disappeared almost completely at times and then again became very marked. Heart-beat during this period varied from 142 per minute at the beginning, to 80 or 86 to 120 at the end. No vomiting occurred.

Following this was a period of marked recovery in which great desire for water was shown. On the following day but slight depression was noticeable. The appetite of the animal was considerably diminished but no vomiting occurred. The next or third day, the animal would not eat, but drank water eagerly. Inactivity was still quite marked. On the fourth day, the dog ate some food and again drank a large quantity of

water. Inactivity was still noticeable. By the fifth day, the dog had fully recovered his appetite. On the sixth day, the animal's hind legs seemed to be paralyzed, salivation was marked, and the breathing labored.

Prostration was more general on the following day, and on the eighth day, the animal was apparently nearly dead. He was then chloroformed and an autopsy performed.

This examination showed nothing specially abnormal. There were no signs of inflammation at the point of injection.

III.—CONCLUSIONS DRAWN FROM THE PRELIMINARY EXPERIMENTS ON THE TOXICITY OF BARIUM BROMID.

The results of our preliminary experiments accord, in a general way, with previous observations by the authors already referred to (foot note, p. 373). The amounts of barium bromid required to produce toxic effects were relatively large, particularly in the first series of experiments. It seemed evident, therefore, that the barium bromid in our radium preparations (in the quantities of the latter which were available for use) would introduce few, if any, complications, especially in the experiments in which the substance was administered per os. This conclusion was confirmed by the later metabolism experiments.

The large quantities of barium bromid that were required per os, to yield toxic effects equal to those produced by subcutaneous injections, emphasize the well-known fact that the soluble salts of the alkali earth metals are absorbed very slowly and very incompletely from the alimentary tract.

Exclusion of bone-ash from the diet in Experiments IV and V did not appear to increase the susceptibility of the animals to the influence of the barium. Our suspicion that sulfate in the bone-ash¹ might have been converted into significant amounts of barium sulfate in the stomach was unfounded. The large quantities of calcium carbonate and phosphate in the bone-ash had no influence on the toxicity of the barium bromid. This was shown conclusively in Experiments IV and V.²

¹ The quantities of water-soluble sulfate in the remaining portions of the food were too small to have any significance in this relation. See page 382.

² Although the soluble bromid of barium did not appear to be absorbed very readily until large doses were administered, the conversion of the barium into the insoluble sulfate would have made absorption of even very large quantities impossible. Orfila, *loc. cit.*, fed small dogs (the weights of the dogs are not given) with as much as 24 grams of barium sulfate, in one dose, without producing any ill effects.

IV.—CONDUCT OF THE METABOLISM EXPERIMENTS.

The metabolism experiments were conducted by the general methods in use in this laboratory.¹

Animals and Environment.—All of the experiments were performed on young healthy dogs, in approximate nitrogenous equilibrium. The dogs under observation were confined in a cage² well adapted to the collection and separation of urine, feces, cast-off hair, and scurf. The structure of the cage permitted free circulation of air. The experiments were conducted in a well lighted and thoroughly ventilated room, the temperature of which was fairly uniform throughout each experiment.

Only such animals as were particularly well suited to the experiments were finally selected after trial periods, *i. e.*, in all cases dogs were used that were lively, playful, and seemed to be contented with their environment and preliminary treatment.

Food.—The food consisted of a mixed diet containing hashed lean beef, lard, cracker-meal, bone-ash,³ and water. The raw meat was preserved in a frozen condition.⁴ The commercial cracker-meal was kept dry in tightly closed jars. Lard was obtained in comparatively small quantities and was always fresh at the time of use. The bone-ash consisted of the thoroughly incinerated, carbon-free, commercial product. Ordinary tap-water was used.

In each of the experiments the dog was given the daily portion of food at 10 A.M.⁵ The solids and water were intimately mixed, and the soupy mass thus formed was eaten with very evident relish.⁶

¹ Mead and Gies, *Amer. Jour. Physiol.*, v, p. 106, 1901; also, Gies and collaborators, *Biochemical Researches*, i, Reprint No. 21, 1903.

² Gies, *Amer. Jour. Physiol.*, xiv, p. 403, 1905. See note, page 410.

³ Gies, *Proc. Amer. Physiol. Soc.*, *Amer. Jour. Physiol.*, x, p. xxii, 1904.

⁴ Gies, *Amer. Jour. Physiol.*, v, p. 235, 1901. Also Gies and collaborators, *Biochem. Researches*, i, Reprint No. 1, 1903. The facilities for refrigeration at the College of Physicians and Surgeons are so complete that meat preserved here by this method is far superior (in its similarity at all times to perfectly fresh beef) to meat that has been dried or treated physically or chemically by any other special process that we know of. Such preservation by refrigeration is especially satisfactory after a relatively thorough mechanical removal of blood and lymph. This removal, by pressure, of the excess of liquid, does not seem to affect the dietary value of the meat in any appreciable degree. See note, page 410.

⁵ Except in the second experiment, during which the day ended at 11.45 A.M.

⁶ The food was usually so ardently desired that it was necessary to use force to prevent the animal from spilling the contents of the dish when it was brought into the cage. This has been observed even when

Periods, Weights.—In the records of our experiments, each day ended at 10 A.M., at which time, just before the food was given, the weight of the dog was taken. The figures for weight in the tables represent, therefore, the weight of the animal at the *end* of the day of record. The daily analytical data also, are recorded for the twenty-four hours ending at 10 A.M. The new periods of record, after preliminary establishment of nitrogenous equilibrium, always began with the day on which the dog was subjected to the new conditions.

Method of Administering Radium and Barium Bromids, per os.—The substance to be administered was always carefully weighed (to a fraction of a milligram) in a platinum scoop on an analytical balance. A small portion of the daily weight of the hashed lean beef (about 25 grams) was then molded in the hand, so as to form a receptacle; to this the substance was transferred. The meat receptacle was molded into a ball, and the ball offered to the dog, which, in every case, ate it eagerly. The first meat-ball was immediately followed by a second, that did not contain any foreign material, so that usually, the dog was just swallowing the first meat-ball without chewing it, when the second one was given, both disappearing quickly. In no case was there any loss of material during the transfer from the balance to the dog's mouth. The dog ate the remainder of the daily diet from an agateware dish, immediately after finishing the first two meat-balls. With very few exceptions, the dog always ate greedily all of the food that was offered him. In each case the diet was amply sufficient to maintain the animal in normal condition.

Subcutaneous Injection of Radium and Barium Bromids.—The carefully weighed quantity of bromid was transferred from the platinum weighing scoop to a small beaker, and dissolved in a small quantity of distilled water (2-4 c.c.). The solution was drawn into a hypodermic syringe. The beaker was rinsed once or twice with small quantities of distilled water. The rinsings were drawn into the syringe. The total volume of the solution injected rarely exceeded 6 c.c. The injection was made on the side of the dog in the lumbar region.

Collection of Excreta.—In general, the excreta were collected in the usual way, as described in a recent paper from this laboratory.¹ The urine was collected as it passed from the body normally; the catheter was not used. Usually, the whole day's urine was allowed to accumulate in the urine-receiver under the cage. At the end of the day (10 A.M.) the urine was transferred to a measuring cylinder then to a stoppered bottle, to which a little powdered thymol was added for preservation.

When there was danger of the dog vomiting, and the vomit running down into, and mixing with, the urine, the urine-receiver was emptied

excessive amounts were fed. The only exceptions, due to large doses of barium bromid, are noted on pages 374, 376, and 385.

¹ Hawk and Gies, *Amer. Jour. Physiol.*, xi, p. 177, 1904.

into a cork-stoppered measuring cylinder, as often as urine was voided. In this way, the urine was almost always collected entirely free from fecal matter and vomit.

The collection of feces, cast-off hair, and scurf, and other general matters relating to the conduct of these experiments were carried out as described in previous papers from this laboratory.¹

Analytic Methods.—The ingredients of the food and the various excreta were analyzed. All determinations of nitrogen were made by the Kjeldahl method; concentrated sulfuric acid, aided by a little cupric sulfate, was used to effect the oxidation.² The digestion was continued for several hours after the mixture had become colorless. Total sulfur and phosphorus were determined by the well-known sodium hydroxid fusion methods of Liebig.

Total sulfate in urine was determined by the usual gravimetric method.³ Total sulfate in feces was determined by first extracting the sample with hot dilute hydrochloric acid, then filtering, washing, and proceeding with the combined filtrate and washings as described in the method for urine. The feces of Experiment I, period 2, which contained considerable amounts of barium (see page 391), were extracted with hot dilute ammonium hydroxid, to prevent solution of any barium phosphate that might be present. This extract was acidified with hydrochloric acid after filtration, and the determination of sulfate carried out as above.

Combined sulfate in urine was determined by the method of Baumann.⁴ In the feces, the combined sulfate was determined by the same method, after the sample (10 grams) had been mixed with about 500 c.c. of water.

Phosphate in urine was determined by the uranium acetate volumetric method.⁵ Phosphate in feces was determined by the same method, after preliminary application of the following process: The feces were incinerated in platinum. The ash was dissolved in strong hydrochloric acid. This solution was then rendered alkaline with ammonium hydroxid, and later re-acidified with acetic acid.

*Purity of the Barium Bromid.*⁶—An analysis of the barium bromid used, showed its percentage content of barium to be 41.05. The theoretical percentage of barium in the compound containing 2 molecules of water of crystallization is 43.57.

The purity of our reagents was always established before their use.

¹ Mead and Gies, Hawk and Gies, *loc. cit*

² Marcuse, *Arch. f. d. ges. Physiol.*, lxiv, p. 232, 1896.

³ Neubauer and Vogel, *Harnanalyse*, pp. 721-22.

⁴ *Ibid.*, p. 724.

⁵ *Ibid.*, pp. 731-33.

⁶ The acid in the stomach is not sufficiently strong to liberate any bromin from the barium bromid. This was conclusively proved by the negative results obtained in an experiment made to remove any doubt on this point.

The recorded analytic data are averages of closely agreeing duplicate results.

V. FIRST METABOLISM EXPERIMENT—INFLUENCE OF BARIUM BROMID ADMINISTERED PER OS.

TABLE I.

PERCENTAGE COMPOSITION OF THE FOOD USED IN THE METABOLISM EXPERIMENTS.¹

PREPARED HASHED LEAN BEEF.

Preparation.		Nitrogen.	Sulfur.	Phosphorus.
No.	Used in Exp't No.	Per cent.	Per cent.	Per cent.
1	1 and 2	3.418	0.2123	0.2029
2	3	3.482	0.1970	0.2253
3	4	3.546	0.2264	0.2239

CRACKER-MEAL.

1	1, 2, and 3	1.649	0.1469	0.1684
2	4	1.608	0.1317	0.2101

BONE-ASH.

1	1, 2, 3, & 4	0.004	0.1496	18.101
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¹To determine the effect of the soluble sulfate in the food, on the barium bromid administered per os, in this experiment, water extracts were made of weighed quantities of meat and cracker-meal and the sulfate was determined in these extracts. The soluble sulfate in the meat amounted to 0.0009 per cent.; that in the cracker-meal amounted to 0.004 per cent. The lard was free from sulfur and the bone-ash contained only very minute and negligible proportions. The dog was fed 90 grams of meat daily, which contained 0.00081 gram of SO_4 , and 35 grams of cracker-meal, which contained 0.0014 gram of SO_4 , or a total of 0.00221 gram of SO_4 . The amount of administered barium bromid was (in the first dose) 0.2310 gram, which contained 41.05 per cent. of barium, or 0.0948 gram of this element. Of this amount, the above quantity of sulfate would have precipitated only 0.00308 gram or 3.25 per cent. of the total amount of barium in this dose.

The small percentage of the barium precipitated in this, the smallest dose given in the experiment, is sufficient ground for the conclusion that the absence of symptoms after administration of the smaller doses was not due to precipitation of the barium as barium sulfate by the soluble sulfates in the food.

TABLE I.—CONTINUED.

LARD

	1 ¹	0 006	trace ²	0 010
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Diet.—The character of the diet was the same throughout the entire experiment.

TABLE II.

COMPOSITION OF THE DAILY DIET.

Ingredients	Hashed Beef	Cracker Meal	Lard	Bone ash	Water	Total
Weight	grams 90 0	grams 35 0	grams 20 0	grams 3 0	grams 210.0	grams 358.0
Nitrogen	3 0762	0 5773	0 0012	0 0001	...	3 6548
Sulfur	0.1911	0 0514	0 0002	0 0045	..	0 2472
Phosphorus	0 1826	0 0589	0 0020	0 5430		0 7865

Preparatory Period.—During the first fourteen days of the experiment (Jan. 18–31, 1905) no analytical work was done. The dog continued, very gradually, to lose weight, from 4.96 kilos (Jan. 18) to 4.67 kilos (Jan. 31). At 10 A.M., Jan. 31, 1905, the daily amount of food was slightly increased, and analytical work was begun on material collected at the end of the fifteenth day, i. e., the day ending Feb. 1, 1905, at 10. A.M.

First Period.—Normal conditions. Maintenance of approximate nitrogenous equilibrium. Days 1–9, Feb. 1–9, 1905. Nitrogenous equilibrium was only approximated, as the animal continued regularly to excrete a little more nitrogen than was ingested, but it seemed best to proceed without further change of diet.

Second Period.—Metabolic influence of barium bromid, administered per os. Days 10–28, Feb. 10–28, 1905. At 10 A.M.

¹In the experience of the workers in this laboratory, nitrogen, sulfur and phosphorus are uniformly absent, except in traces, from the kind of lard used. For this reason, the nitrogen, sulfur, and phosphorus were determined in only one sample of lard, and these figures were used for all.

²The figure actually obtained was 0.00086 per cent.

Feb. 9, 1905, the dog was given the first dose of barium bromid.¹ Diarrhœa² soon followed after the first dose of barium bromid per os. Beginning with 0.2310 gram (50 mg. per kilo), the amount was gradually increased daily to 0.8085 gram, (175 mg. per kilo). From the twentieth to the twenty-eighth day the diarrhœa was so much greater than from the tenth to the nineteenth day, that this period was divided into two sub-periods; the first (Days 10-19) in which there was mild diarrhœa and intestinal irritation, and the second sub-period (Days 20-28) in which these tendencies were more pronounced. In this way any variation in the elimination of nitrogen, sulfur, and phosphorus in the feces, which might be caused by the increased dosage of barium bromid during this second sub-period, could be determined.

First Sub-period. Days 10-19.—Slight diarrhœa continued throughout the entire period. There was slight vomiting on the fourteenth day. At the end of this period, the dog was as active and lively as usual, nothing abnormal in his appearance or behavior being observed.

Second Sub-period. Days 20-28.—There was more pronounced diarrhœa, especially in the latter part of the period. On the twenty-fifth day, there was no diarrhœa, the dog was lively and normal. At the end of the twenty-eighth day, the experiment was discontinued, and no analytical work was done on the excreta collected on the twenty-ninth day. The dog was very sick, and vomited several times. On these occasions the excreta could not be collected separately.

Day 28 (10 A.M. Feb. 27—10 A.M. Feb. 28).—The dog was less active than usual and looked sick. There was mucous diarrhœa and frequent vomiting; the vomit containing meat-fibres (seen under the microscope). During the day, 153 c. c. of

¹ The barium bromid (Kahlbaum's) used in the first and second metabolism experiments (and in the preliminary experiments) contained two molecules of water of crystallization. It was used in this condition, so that all of the recorded weights of barium bromid are weights of the salt containing this quantity of water. The recorded weight multiplied by 0.8919 gives the weight of anhydrous barium bromid.

² The very strong purgative action of barium salts had been observed (with very few exceptions) by all of the previous investigators mentioned on page 373, footnote.

urine, containing no fecal matter or vomit, were collected and reserved for the purpose of testing for barium.

Day 29 (10 A.M., Feb. 28—Mar. 1.)—At 11 A.M. the dog was growing very sick, and vomited froth repeatedly. There was loss of power in the hind legs, and muscular twitching of the abdomen and leg muscles, followed by great depression and gradual general prostration. The dog repeatedly refused food. During the day the urine-receiver contained a mixture of fecal matter, urine, and vomited material which could not be used for analytical purposes. At 5.45 P.M. the heart-beat was 140, regular and strong; respiration, 16. At 6.35 P.M. the dog's breathing was very labored, as if suffocating. At 8.15 P.M. the dog was found dead, the body not yet cold.

Autopsy 8.30.—The intestines were constricted and congested. The pancreas was red¹. The bladder was collapsed, only 1.8 c.c. of urine were obtainable by pressure. There was no urine in the receiver since 10.30 A.M. Nearly ten hours had elapsed, during which only 1.8 c.c. of urine were excreted. The heart was distended in full diastole. The stomach was empty and normal in appearance.

Analytical Results.—The analytical data for Experiment I are given in Tables I–VI.

Discussion of Results.—Changes in weight.—The dog was slowly losing weight when the experiment began (see *Preparatory Period*, p. 383). The figures in Table III show that the barium bromid did not materially affect the dog's weight. The changes in weight before and during the barium feeding are practically the same, in magnitude and direction. The increased elimination of water in the feces partly accounts for the slight decline in weight, although the increase of fecal water was counterbalanced to a certain extent by the decrease in urinary water.

Fluctuations in the Volume of Urine.—During the normal period, there were no marked variations in the daily volume of urine. During the latter part of the barium period, the volume was diminished. Throughout the entire second period, the daily average volume diminished, probably because of diarrhœa, more water being excreted in the feces than during the first period.

¹This was also noticed in the dog used in Experiment II, page 395.

TABLE III.
DAILY RECORDS OF THE FIRST METABOLISM EXPERIMENT.

First period.—Maintenance of normal approximate nitrogenous equilibrium. Feb. 1-9, 1905.									
Day No.	Body Weight.	Barium Bromid		Urine.				Period Average to Date.	
		Total Dose	Mg. per Kilo	Volume.	Specific Gravity.	Nitrogen.	Total Daily Sulfate.	Volume.	Nitrogen.
		grams.		c.c.		grams.	grams.	c.c.	grams.
1	4.65	—	—	239	1018	3.9184	0.5701	239	3.9184
2	4.67	—	—	206	1018	3.7516	0.5329	222	3.8350
3	4.67	—	—	246	1017	3.8825	0.5684	230	3.8568
4	4.67	—	—	224	1018	3.8825	0.5625	229	3.8587
5	4.64	—	—	248	1018	3.9050	0.5599	233	3.8680
6	4.65	—	—	232	1017	3.6433	0.5372	232	3.8305
7	4.64	—	—	228	1017	3.7475	0.5489	232	3.8187
8	4.62	—	—	249	1018	4.1662	0.6155	234	3.8621
9	4.62	—	—	234	1017	3.7970	0.5661	234	3.8549
Second period.—Metabolic influence of barium bromid administered per os. First sub-period 1—Feb. 10-19, 1905.									
10	4.58	0.2310	50	258	1019	3.9074	0.5528	238	3.9074
11	4.57	0.2310	50	226	1017	3.6640	0.5292	242	3.7857
12	4.57	0.2310	50	230	1017	3.7591	0.5402	228	3.7768
13	4.56	0.2310	50	254	1016	3.7219	0.5338	242	3.7631
14	4.51	0.2310	50	256	1016	3.7619	0.5201	245	3.7629
15	4.54	0.3465	75	201	1017	3.5155	0.5127	238	3.7219
16	4.53	0.3696	80	210	1016	3.4701	0.4859	234	3.6857
17	4.52	0.3927	85	237	1016	3.4993	0.3505	234	3.6624
18	4.54	0.4158	90	200	1017	3.4225	0.4614	230	3.6357
19	4.51	0.4389	95	242	1016	3.5595	0.4628	231	3.6281
Second Period.—Second sub-period—Feb. 20-28, 1905.									
20	4.50	0.4620	100	235	1016	3.4566	0.4658	232	3.6125
21	4.42	0.5082	110	252	1020	4.1363	0.4644	233	3.6562
22	4.31	0.5082	110	210	1022	3.5950	0.4902	232	3.6445
23	4.33	0.5082	110	186	1020	4.5693	0.6266	228	3.7106
24	4.36	0.5313	115	168	1018	3.5190	0.5001	224	3.6978
25	4.38	0.5544	120	180	1017	3.5003	0.4664	222	3.6858
26	4.42	0.6066	130	173	1017	3.2854	0.4162	219	3.6623
27	4.45	0.6930	150	184	1017	3.3425	0.4854	217	3.6501
28	4.26	0.8685	175	227	1016	3.3149	0.4666	217	3.6272
Feces. ¹ Dry Weight. grams.									
1									6.2
2									7.5
3									5.5
4									7.0
5									—
6									12.8
7									7.6
8									5.8
9									
10									9.8
11									7.5
12									3.8
13									9.4
14									7.4
15									6.5
16									8.3
17									8.0
18									7.9
19									5.2
20									11.1
21									7.8
22									9.2
23									11.1
24									9.0
25									8.5
26									8.0
27									7.9
28									15.2

¹ Blanks under feces in this and all subsequent tables signify no elimination.

² See pages 383, 384.

TABLE IV.
METABOLISM OF SULFUR IN SULFATE.

PERIOD FIGURES.

Occurrence	First Period, Days 1-9			Second Period, Days 10-19			Second Period, Days 20-28		
	S in Total SO ₄	S in Combined SO ₄	S in Pre-formed SO ₄	S in Total SO ₄	S in Combined SO ₄	S in Pre-formed SO ₄	S in Total SO ₄	S in Combined SO ₄	S in Pre-formed SO ₄
	grams	grams	grams	grams	grams	grams	grams	grams	grams
Urine	1.6841	0.0805	1.6036	1.6992	0.0861	1.6131	1.4863	0.0578	1.4285
Feces	0.0449	0.0087	0.0362	0.0074	0.0038	0.0036	0.0048	0.0039	0.0008
Cage washings	0.0119	—	0.0119	0.0277	—	0.0277	0.0249	—	0.0249
Total	1.7409		1.6517	1.7343		1.6444	1.5160		1.4542

DAILY AVERAGES.

Urine	0.1871	0.0090	0.1781	0.1699	0.0086	0.1613	0.1651	0.0064	0.1587
Feces	0.0050	0.0010	0.0040	0.0007	0.0004	0.0003	0.0005	0.0004	0.0001
Cage washings	0.0013	—	0.0013	0.0028	—	0.0028	0.0028	—	0.0028
Total	0.1934		0.1834	0.1734		0.1644	0.1684		0.1616

METABOLISM OF PHOSPHORUS IN PHOSPHATE.

	P in PO ₄ Period Figures	P in PO ₄ Daily Averages	P in PO ₄ Period Figures	P in PO ₄ Daily Averages	P in PO ₄ Period Figures	P in PO ₄ Daily Averages
Urine	1.0743	0.1194	1.0661	0.1066	0.7843	0.0871
Feces	2.3252	0.2584	2.8659	0.2866	2.7713	0.3079
Cage washings	0.0026	0.0003	0.0128	0.0013	0.0526	0.0059
Total	3.4021	0.3781	3.9448	0.3945	3.6082	0.4009

TABLE V.

ANALYTIC TOTALS AND AVERAGES.

Elements	Nitrogen		Sulfur		Phosphorus	
Periods	I 9 days	II 19 days	I 9 days	II 19 days	I 9 days	II 19 days
	grams	grams	grams	grams	grams	grams
Food	32.8930	69.4400	2.2248	4.6968	7.0785	14.9430
Excretions	37.4210	77.2500	2.4760	4.7499 (5.5474) ¹	7.0681	15.3866
Balance	-4.5280	-7.8100	-0.2512	-0.0531 (-0.8506)	+0.0104	-0.4436

AVERAGE DAILY BALANCES.

	-0.503	-0.411	-0.0279	-0.0028 (-0.0448)	+0.0012	-0.0233
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TOTAL AMOUNTS IN EXCRETIONS.

Urine	34.6940	68.9165	2.0438	3.9061	2.2560	3.8770
Cage w'sh'gs	0.2744	0.7658	0.0193	0.1001	0.0113	0.0932
Feces	2.0457	2.6244 ² 3.7419	0.3066	0.2051 ² 0.2314 (0.5050) (0.7290)	4.7580	5.7510 ² 5.5724
Hair	0.4068	1.2016	0.1063	0.3072	0.0428	0.0930
Total	37.4209	77.2502	2.4760	4.7499 (5.5474)	7.0681	15.3866

AVERAGE DAILY AMOUNTS IN EXCRETIONS.

Urine	3.8550	3.6272	0.2271	0.2056	0.2507	0.2040
Cage wsh'gs	0.0305	0.0403 ³	0.0021	0.0053	0.0013	0.0049
Feces	0.2273	0.2624 0.4158	0.0341	0.0205 ³ 0.0257 (0.0505) (0.0810)	0.5287	0.5751 ³ 0.6192
Hair	0.0452	0.0632	0.0118	0.0162	0.0048	0.0050
Total	.41580	4.0657	0.2751	0.2501 (0.2920)	0.7855	0.8099

DAILY AMOUNTS IN FOOD.

	3.6548	0.2472	0.7865
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¹ The figures in parentheses are explained on pages 389 and 390.² Totals for 10 and 9 days, respectively. See page 384.³ These figures are sub-period averages for 10 and 9 days, respectively.

TABLE VI.

QUANTITIES OF FECES, CAST-OFF HAIR, AND SCURF FOR EACH PERIOD.

Periods	I 9 days	II 19 days
FECES.		
Total weight, dry.	grams 59.4	grams 161.8
Average daily weight, dry.	6.6	8.5
CAST-OFF HAIR AND SCURF.		
Total weight	4.0	10.7
Average daily weight	0.44	0.6

Specific Gravity of the Urine.—The variations in specific gravity were slight. The higher figures of the twenty-first, twenty-second, and twenty-third days were probably due to small quantities of semi-fluid fecal matter washed into the urine-receiver, at the time when the dog had marked diarrhoea (see p. 384).

Nitrogenous Catabolism.—The figures in Tables III and V for nitrogen show that the nitrogen balance was little affected by the 8.2929 grams of barium bromid administered during the nineteen days of the second period. There was an increase in the fecal nitrogen in both parts of the second period, due to diarrhoea, and a corresponding decrease in the urinary nitrogen.

Sulfur Catabolism.—The results for sulfur catabolism are practically the same as those for nitrogen.

To determine total sulfur in feces, the dried, finely powdered fecal matter was fused in a silver crucible with sodium hydroxid and a little potassium nitrate, according to the usual method of Liebig. The fused mass was transferred to a casserole, dissolved in water, and acidified with hydrochloric acid. If barium had been present in the feces, it would have been precipitated at this point, in part at least, as sulfate. The liquid was evaporated to dryness, the dry residue was moistened with hydrochloric acid, taken up with water (about 100–150 c.c. usually), and filtered. To the clear filtrate, barium chlorid solution was added and the usual procedure followed. Obviously, the total sulfur found was low by the amount precipitated as barium sul-

fate, after acidifying and before filtering. The residue remaining on the filter paper, consisting of small quantities of barium sulfate, silver chlorid, etc., was rejected.

During the second period, the dog received a total of 8.2929 grams of barium bromid, in the food. It is possible that practically all of the barium was precipitated as sulfate, and carried out 0.7975 grams of sulfur in the feces. The figures for sulfur in feces (Table V) which are not in parentheses are the actual analytical figures obtained. The figures in parentheses show the amounts obtained plus the amounts calculated from the known weights of barium bromid administered to the dog during the first and second parts of the second period. The total amount of sulfur, 0.7975 grams, so calculated and added to the total amount found in the feces, while fairly large in absolute amount, makes a relatively small difference in the sulfur balance.

Sulfate-Sulfur Catabolism.—There was a slight decrease in the amount of sulfate sulfur eliminated in the urine during the second period, which showed that this was very slightly affected, if at all, by the barium bromid feeding (see Tables III and IV).

Phosphorus Catabolism.—The figures of Table V show that no marked changes in phosphorus catabolism took place.

Phosphate-Phosphorus Catabolism.—This was not affected.

Feces, Cast-off Hair, and Scurf.—In all probability, the more marked diarrhoea during the second period caused the increased elimination of nitrogen, sulfur, and phosphorus in the feces, and the corresponding decrease in the urine shown by the average daily amount excreted, in Tables III and V. No changes in the quality or quantity of the cast-off hair and scurf were observed.

Elimination of Barium Bromid.—On the twenty-ninth day of the barium bromid feeding experiment a fraction of the day's urine was obtained free from feces. Forty-four cubic centimeters of this urine were evaporated to dryness, then fused with sodium hydroxid and potassium nitrate to a clear liquid. After cooling, the fused mass was taken up with dilute hydrochloric acid. On standing twelve hours no precipitate was visible. The solution was then evaporated to dryness and the residue fused with sodium carbonate. This mass on cooling was dissolved in water and filtered. The precipitate was dissolved in a very small quantity of hydrochloric acid and this solution tested for

barium in the usual manner with solutions of calcium sulfate and potassium dichromate and by means of the spectroscope. All these tests were negative.

A test made on some of the combined feces for the second period of the experiment in a similar manner, showed the presence of barium in considerable quantity.

VI. SECOND METABOLISM EXPERIMENT; INFLUENCE OF BARIUM BROMID, INJECTED SUBCUTANEOUSLY.

Diet.—The diet was the same daily throughout the entire experiment.

TABLE VII.

COMPOSITION OF THE DAILY DIET.

Ingredients.	Hashed Beef.	Cracker Meal.	Lard.	Bone-ash.	Water.	Total
Weight.	grams. 117.0	grams. 42.0	grams. 20.0	grams. 5.0	grams. 260.0	grams. 444.0
Nitrogen	3.9991	0.6928	0.0012	0.0002	—	4.6933
Sulfur	0.2484	0.0617	0.0002	0.0075	—	0.3178
Phosphorus	0.2374	0.0707	0.0020	0.9051	—	1.2152

Preparatory Period.—During this period, lasting 18 days, March 4–21, 1905, the dog's weight changed from 6.71 kilos at the beginning to 6.55 kilos at the end. On March 21, 1905, at 11.45 A.M., the diet was increased; the new diet was continued throughout the entire experiment. At the same time, on this day, the collection of excreta for analytical work was begun. During this experiment, the experimental day began at 11.45 A.M., and ended at 11.45 A.M. on the following day.

First Period.—*Normal conditions. Maintenance of approximate nitrogenous equilibrium.* Days 1–7, March 22–28, 1905. Nitrogenous equilibrium could not be established perfectly without a change of diet, but, as in the previous experiment, the difference between the ingested and excreted nitrogen was comparatively slight.

Second Period.—*Metabolic influence of barium bromid, injected subcutaneously.* Days 8–19, March 29–April 9, 1905. On March 28, 1905, at 4.23 P.M., the dog was given the first injection of barium bromid, 0.0131 grams (2 mg. per kilo); the

TABLE VIII.

DAILY RECORDS OF THE SECOND METABOLISM EXPERIMENT.

First Period.—Maintenance of normal approximate nitrogenous equilibrium. March 22–28, 1905.

Day No.	Body Weight kilos	Barium Bromid.		Urine.							Feces. Dry Weight. grams.
		Total Dose grams.	Mg. per Kilo.	Volume. c. c.	Specific Gravity.	Nitrogen. grams.	Total Daily Sulfate. grams.	Period Average to Date.			
								Volume. c. c.	Nitrogen. grams.	Total Daily Sulfate. grams.	
1	6.53	—	—	312	1016	4.4470	0.5833	312	4.4470	0.5833	9.6
2	6.54	—	—	297	1016	4.1918	0.4861	304	4.3194	0.5347	8.4
3	6.53	—	—	310	1016	4.6829	0.6178	306	4.4404	0.5624	8.7
4	6.54	—	—	295	1016	4.5881	0.5830	303	4.4774	0.5676	11.8
5	6.55	—	—	307	1016	4.2943	0.5595	304	4.4408	0.5659	13.1
6	6.55	—	—	308	1015	4.6828	0.6189	305	4.4811	0.5748	7.1
7	6.57	—	—	291	1016	4.4000	0.5134	303	4.4696	0.5600	9.6

Second period.—Metabolic influence of barium bromid injected subcutaneously. March 29–April 9, 1905.

8	6.62	0.0131	2	283	1016	4.3383	0.6162	283	4.3338	0.6162	11.9
9	6.63	0.0131	2	287	1016	3.9388	0.5247	285	4.1385	0.5705	13.5
10	6.63	0.0131	2	268 ¹	1015	3.6072	0.4351	279	3.9614	0.5253	9.8
11	6.63	0.0263	4	317	1014	3.9105	0.5047	289	3.9487	0.5202	5.8
12	6.60	0.0394	6	312	1014	4.2831	0.5214	293	4.0156	0.5204	11.9
13	6.59	0.0526	8	315	1015	4.1640	0.5116	297	4.0403	0.5190	8.6
14	6.63	0.0657	10	263	1017	4.2236	0.5263	292	4.0665	0.5200	13.3
15	6.64	0.0788	12	317	1015	4.6725	0.5665	295	4.1422	0.5258	8.4
16	6.67	0.0920	14	278	1017	4.5684	0.5808	293	4.1896	0.5319	4.6
17	6.61	0.1182	18	345	1015	4.5318	0.5774	298	4.2238	0.5365	15.9
18	6.59	0.1642	25	305	1016	4.3515	0.5182	299	4.2354	0.5348	7.6
19	6.57	0.2628	40	261	1019	4.6721	0.5506	296	4.2718	0.5361	9.0
20	Died	0.4270	65	95							

¹ About 20 c.c. of urine were accidentally spilled on the sliding shelf under the cage; none of the urine fell to the floor. Most of the urine was removed from the shelf with a clean sponge, the shelf and the sponge were washed several times with water, and the washings added to the cage-washings for this period. The total loss, though indeterminate, was probably very slight. The volume recorded (268 c.c.) was the volume actually obtained.

amount injected was gradually increased daily to 0.4270 grams (65 mg. per kilo). The behavior and appearance of the dog were generally normal. Occasionally, during this period, the dog showed signs of discomfort, weakness in the hind legs, and general fatigue. These symptoms lasted but a short time; they appeared and disappeared irregularly. On the twentieth day (April 9, 1905, at 11.45 A.M. to April 10, 1905, 11.45 A.M.) up to 6.55 P.M., the dog's behavior was entirely normal. Nothing was observed which would indicate that death would occur within the next twenty-four hours. On Monday, April 10, 1905, at 7.50 A.M., the dog was found dead; the body still warm. The animal had vomited abundantly during the night.

TABLE IX.

METABOLISM OF SULFUR IN SULFATE.

PERIOD FIGURES.

Occurrence	First Period, Days 1-7.			Second Period, Days 8-19		
	S in Total SO ₄	S in Combined SO ₄ .	S in Preformed SO ₄ .	S in Total SO ₄ .	S in Combined SO ₄	S in Preformed SO ₄ .
	grams	grams	grams.	grams	grams	grams
Urine	1.3464	0.0809	1.2655	2.1663	0.1325	2.0338
Feces	0.1341	0.0046	0.1295	0.1966	0.0098	0.1868
Cage washings	0.0078	—	0.0078	0.0233	—	0.0233
Total	1.4883		1.4028	2.3862		2.2439

DAILY AVERAGES.

Urine	0.1923	0.0116	0.1807	0.1805	0.0110	0.1695
Feces	0.0192	0.0007	0.0185	0.0164	0.0008	0.0156
Cage washings	0.0011	—	0.0011	0.0019	—	0.0019
Total	0.2126		0.2003	0.1988		0.1870

METABOLISM OF PHOSPHORUS IN PHOSPHATE.

	P in PO ₄ Period Figures	P in PO ₄ Daily Averages	P in PO ₄ Period Figures	P in PO ₄ Daily Averages
Urine	0.9723	0.1389	1.5548	0.1295
Feces	2.8910	0.4130	4.9920	0.4160
Cage washings	0.0006	0.0001	0.0150	0.0013
Total	3.8639	0.5520	6.5618	0.5468

TABLE X.

ANALYTIC TOTALS AND AVERAGES.

Elements.	Nitrogen.		Sulfur.		Phosphorus.	
Periods.	I 7 days.	II 12 days.	I 7 days.	II 12 days.	I 7 days.	II 12 days.
	grams.	grams.	grams.	grams.	grams.	grams.
Food	32.8531	56.3196	2.2246	3.8136	8.5064	14.5824
Excretions	33.5584	55.5004	2.2052	3.7292	8.2557	14.4072
Balance	-0.7053	+0.8192	+0.0194	+0.0844	+0.2507	+0.1752

AVERAGE DAILY BALANCES.

	-0.1008	+0.0683	+0.0028	+0.0070	+0.0358	+0.0149
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TOTAL AMOUNT IN EXCRETIONS.

Urine	31.2869	51.2618	1.7759	2.8547	2.0986	3.2897
Cage washings	0.1024	0.4290	0.0159	0.0554	0.0090	0.0450
Feces	1.8272	3.0907	0.3543	0.6769	6.1206	11.0503
Hair	0.3419	0.7189	0.0591	0.1422	0.0275	0.0222
Total	33.5584	55.5004	2.2052	3.7292	8.2557	14.4072

AVERAGE DAILY AMOUNTS IN EXCRETIONS.

Urine	4.4696	4.2718	0.2537	0.2379	0.2998	0.2741
Cage washings	0.0146	0.0357	0.0023	0.0046	0.0013	0.0038
Feces	0.2610	0.2576	0.0506	0.0564	0.8744	0.9209
Hair	0.0488	0.0599	0.0084	0.0118	0.0039	0.0019
Total	4.7940	4.6250	0.3150	0.3107	1.1794	1.2007

DAILY AMOUNTS IN FOOD.

	4.6933	0.3178	1.2152
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TABLE XI.

QUANTITIES OF FECES, CAST-OFF HAIR AND SCURF FOR EACH PERIOD.

Periods.	I 7 days.	II 12 days.	
FECES.			
	grams.	grams.	
Total weight, dry	68.3	120.3	
Average daily weight, dry.	9.76	10.02	
CAST-OFF HAIR AND SCURF.			
Total weight	3.2	6.7	
Average daily weight	0.46	0.56	

Autopsy.—April 10, 1905, 12-1, P.M. Lungs congested. Heart distended. Liver and kidneys apparently normal. Pancreas uniformly red,¹ with marked congestion in spots. Intestines normal. No fluid in abdominal cavity. Venous blood black. Spleen and gall-bladder normal. Tissue around point of last injection, dark-blue, like a bruise. Stomach entirely empty. Blood, in large vessels, fluid; clotted a few minutes after removal. Black blood in heart, partly clotted. Slight inflammation, only superficial, under skin on right side at point of injection. Left side: superficial inflammation extending along abdomen. No necrosis.

Analytical Results.—The analytical results for the second experiment are recorded in Tables VII-XI.

Discussion of Results.—The figures of Table VIII show that the 1.3663 grams of barium bromid injected during the twelve days in the second period had no marked effect on the weight of the dog, the daily volume of urine, or its specific gravity. The occasional increase in the daily volume of urine in the second period is probably due to the dog's nervous excitement just before the injection. The diarrhoea² which attended the barium feeding was absent here, the daily quantity and consistency of the feces remained practically unchanged. The same is true of the cast-off hair and scurf.

¹ This was also noticed in the dog used in Experiment I, p. 385.

² See page 384.

A test made on 30 grams of feces from the second period of this experiment, by the method outlined in Experiment I, p. 390, showed the absence of barium¹ in any such quantities as could be detected by the delicate tests applied.

Nitrogenous Catabolism.—The figures in Tables VIII and X show a continued decrease in the quantity of nitrogen eliminated. They suggest that there was, perhaps, a slight inhibition of the catabolic processes, by the barium bromid injected.

Sulfur Catabolism.—The results for sulfur are, in general, the same as those for nitrogen. It may be argued, that the apparent decrease in sulfur catabolism shown in Table X is possibly due to the precipitation of sulfur as barium sulfate by the barium bromid injected, the sulphate being retained by the dog. The total quantity of barium bromid injected during the second period, 1.3663 grams, would precipitate 0.1314 gram of sulfur, forming 0.9569 gram of barium sulfate. Adding the 0.1314 gram of sulfur to the 3.7292 grams in the excreta, the calculated average daily balance would then be -0.0040 gram. The influence of the barium bromid must have been slight, in this connection, as the difference between an average daily balance of $+0.0028$ and -0.0040 gram indicates.

Sulfate-sulfur Catabolism.—This was not affected.

Phosphorus and Phosphate-Phosphorus Catabolism.—No marked effects were observed.

VII. GENERAL REMARKS ON THE METABOLIC EFFECTS OF BARIUM BROMID.

That barium bromid, when administered per os in fairly large

¹ Bary, *loc. cit.*, p. 118, Experiment VIII.—A rabbit which weighed 0.8 kilo was given a subcutaneous injection of 3 c.c. of 2 per cent. barium chlorid solution (75 mg. barium chlorid per kilo). Barium was found in the rabbit's feces, but the quantity found was not mentioned. Bary recorded twenty experiments in which barium chlorid was injected subcutaneously into cats (5), rabbits (5), dogs (5), hens (2), a guinea pig, a white rat, and a pigeon. The amounts injected varied between 10 and 100 mg. per kilo (equivalent to 16 to 160 mg. barium bromid per kilo). In only one case (the above-mentioned) was barium found in the feces. The rabbit was given only one injection (75 mg. per kilo) of barium chlorid; death occurred nine and one-half hours later. This amount is equivalent to 120 mg. barium bromid per kilo, which is almost twice as large as the last and largest dose of barium bromid injected during this experiment (see Table VIII, p. 392).

quantities and for long periods, exerts relatively little metabolic influence, was shown by the results of the first metabolism experiment. The principal effects noticed during that experiment were decreased excretion of urinary water and increased elimination of water and solids per rectum. Under the initial conditions of that experiment, little or no absorption of barium occurred. Excessive quantities of the barium compound which were fed near the end of the experiment, however, finally overcame the resistance offered by the alimentary epithelium, appreciable absorption occurred, and death quickly ensued.

In view of the marked toxicity of barium bromid that was manifested in the second series of our preliminary experiments (Experiments VI-VIII, page 376), it was very surprising to find that the introduction of comparatively large and frequently repeated amounts subcutaneously had little or no effect on metabolism or even on intestinal elimination. That this experiment was exceptional seems certain from our own previous results and from the observations of MacCallum¹ and others. At the same time it may be said, in anticipation, that the results of the fourth metabolism experiment (page 407), in which a radium barium preparation was used, accord, in a general way, with the results of the second metabolism experiment.

The same barium bromid preparation (Kahlbaum's) was used in the preliminary and in the first and second metabolism experiments; hence, no variations which might occur because of differences in the preparations used were possible (see page 384 footnote).

That only traces of barium were absorbed in these experiments, was shown by the fact that barium could not be detected in the urine² of the first metabolism experiment (see Day 29, p. 385; also Elimination of barium bromid, p. 390) or in the feces of the second metabolism experiment (p. 396).

¹ *Loc. cit.*

² Bary, *loc. cit.*, p. 121, Experiment XV.—A female dog which weighed 21.5 kilos was given a subcutaneous injection of 4 c.c. of 5 per cent. barium chlorid solution (10 mg. barium chlorid, equivalent to 16 mg. barium bromid, per kilo). Five hours after the injection, the dog was catheterized, and 60 c.c. of clear urine were obtained. Barium was present in the urine, but the author does not mention the quantity found. "Bei der Veraschung des Urins, war eine deutliche Menge von Baryum

VIII. THIRD METABOLISM EXPERIMENT. INFLUENCE OF RADIUM BROMID (CONTAINING BARIUM BROMID) ADMINISTERED PER OS.

Diet.—The diet was the same daily, throughout the entire experiment.

TABLE XII.
COMPOSITION OF THE DAILY DIET.

Ingredients.	Hashed Beef.	Cracker Meal.	Lard.	Bone-ash.	Water.	Total.
Weight.	grams. 110.0.	grams. 40.0.	grams. 19.0.	grams. 5.0.	grams. 244.0.	grams. 418.0
Nitrogen	3.8305	0.6598	0.0012	0.0002	—	4.4917
Sulfur	0.2167	0.0588	0.0002	0.0075	—	0.2832
Phosphorus	0.2478	0.0674	0.0019	0.9051	—	1.2222

Preparatory Period.—A preparatory period of five days on the above diet was sufficient to get the dog into a condition approximating nitrogenous equilibrium. During this time the weight of the animal fluctuated between 6.52 and 6.58 kilos. At 10 A.M., April 28, 1905, the collection of material for analytical work was begun.

First Period.—Normal conditions. Maintenance of approximate nitrogenous equilibrium. Days 1-5, April 29-May 3, 1905. The animal was further accustomed to its environment.

Second Period.—Metabolic influence of radium bromid (containing barium bromid), administered per os. Days 6-17, May 4-15, 1905. Three radium bromid preparations were used in this experiment; they were of 240, 1,000 and 10,000 activity respectively. At 10 A.M., May 3, the dog was given the first dose, 0.1187 gram (18 mg. per kilo), of the 240 activity radium bromid. This preparation was administered in gradually in-

vorhanden, welche *spectroskopisch* sicher gestellt war." This is the only experiment (out of twenty similar ones, see p. 396) in which barium was found in the urine. In all the other experiments, the weight of barium chlorid injected, per kilo, was larger than in this one, varying from 15 to 100 mg. (equivalent to 24 to 160 mg. barium bromid) per kilo. In Bary's Experiment VIII, in which a rabbit was given 75 mg. barium chlorid (equivalent to 120 mg. barium bromid) per kilo, subcutaneously, barium was found in the feces, but no record was made of the presence or absence of barium in the rabbit's urine. *Neumann, loc. cit.*, found barium in the urine of a dog which had received 0.10 gram of barium chlorid (equivalent to 0.160 gram of barium bromid) per os, but the amount of barium found was not mentioned. *Santi, Chem. Centralblatt*, i, p. 198, 1904, found that in cases of barium poisoning most of the barium left the body in the feces; the amount of barium in the urine was too small to be determined quantitatively.

creased quantities every day for six days, the dog being given the last dose, 0.3152 gram (47 mg. per kilo) of the 240 activity radium bromid at 10 A.M., May 8, 1905. At 10 A.M., May 9, the dog was given the first dose, 0.0506 gram (8 mg. per kilo), of the 1,000 activity radium bromid. The same preparation was administered on the two following days, the doses being 0.1011 and 0.0980 gram (15 mg. per kilo). At 10 A.M., May 12, the dog was given the first dose, 0.0506 gram (4 mg. per kilo), of the 10,000 activity radium bromid. The same preparation was administered on the two following days; the doses being 0.0490 and 0.0515 gram (7 and 8 mg. per kilo, respectively). The amounts of the radium preparations administered are also indicated in Table XIII, p. 400.

The dog's behavior and appearance during this period were normal.

Third Period.—After-period. Days 18–22, May 16–20, 1905. Observation and analytic work were continued during this period, for the purpose of noting after effects, if any were exhibited. Apparently there was none. The dog remained in the cage under observation, until June 3, 1905.

Analytical Results.—The analytical results for the third experiment are recorded in Tables XII–XVI.

Discussion of Results.—The figures in Tables XIII and XVI show that a total administration of 1.3916 grams of the radium bromid preparations produced no marked effects. There were no unusual variations in the quantity or quality of the excreta.

The feces eliminated after the radium feeding and up to June 1, 1905, were relatively highly radio-active. The feces collected after June 1, were not radio-active.

Post-mortem examination of the dog, for the purpose of finding, if possible, the paths of distribution and elimination of radio-active material, gave the following results: the lungs, gall-bladder, spleen, cæcum, sigmoid flexure, testicles, muscles, brain and spinal cord, blood serum and clot, were not radio-active; the liver, kidneys, pancreas, stomach, and post-mortem urine were slightly radio-active.¹

¹ For these results, we are indebted to our colleague, Dr. G. M. Meyer. They were obtained in connection with some experiments now in progress in this laboratory. A more detailed description of these, and similar results in other connections, will be given in a paper soon to appear, by Dr. Meyer. We wish to thank him for his cordial co-operation.

TABLE XIII
DAILY RECORDS OF THE THIRD METABOLISM EXPERIMENT.

First period—Maintenance of normal approximate nitrogenous equilibrium, April 29–May 3, 1905.

Day No.	Body Weight, kilos	Radium Bromid.		Urine.						Feces.	
		Total Dose	Active-ity.	Mg. per Kilo Body Weight.	Specific Gravity.	Volume.	Nitrogen.	Total Daily Sulfate.	Period Average to Date.		
									Volume.		Nitrogen.
		grams				c.c.	grams	grams	c.c.	grams	grams
1	6.62	—	—	—	1017	225	3.1944	0.3290	225	3.1944	0.3290
2	6.63	—	—	—	1016	240	3.3403	0.3691	232	3.2673	0.3491
3	6.62	—	—	—	1017	229	3.2865	0.3546	231	3.2737	0.3509
4	6.61	—	—	—	1016	265	3.4713	0.3862	240	3.3231	0.3597
5	6.63	—	—	—	1018	219	3.1465	0.3811	236	3.2878	0.3640
											grams
											—
											6.1
											12.8
											2.3
											4.3

Second period—Metabolic influence of radium bromid, administered per os, May 4–15, 1905.

6	6.64	0.1187	—	18	1016	237	3.2847	0.4111	237	3.2847	0.4111	12.7
7	6.61	0.1048	—	16	1016	237	3.2542	0.3849	237	3.2694	0.3980	16.6
8	6.57	0.1076	—	16	1016	252	3.4890	0.4016	242	3.3426	0.3992	23.5
9	6.56	0.1813	0.4	27	1015	235	3.3227	0.3614	240	3.3376	0.3898	14.1
10	6.59	0.1888	—	28	1015	243	3.5376	0.3619	241	3.3776	0.3842	—
11	6.46	0.3152	—	47	1016	295	3.6491	0.4425	250	3.4229	0.3939	6.7
12	6.49	0.0506	0.0	8	1015	249	3.5505	0.4077	250	3.4411	0.3959	8.3
13	6.49	0.1011	0.0	15	1017	250	3.6836	0.4445	250	3.4714	0.4019	9.2
14	6.50	0.0980	0.1	15	1017	234	3.5780	0.4440	248	3.4833	0.4064	4.6
15	6.53	0.0250	0.0	4	1018	229	3.7006	0.4773	246	3.5050	0.4137	8.9
16	6.54	0.0490	0.0	7	1017	254	3.8198	0.5068	247	3.5336	0.4222	6.1
17	6.57	0.0515	0.1	8	1017	238	3.6850	0.5086	246	3.5462	0.4295	12.2

Third period—After period, May 16–20, 1905.

18	6.61	—	—	—	1017	240	3.7736	0.5099	240	3.7736	0.5099	—
19	6.65	—	—	—	1018	248	3.8516	0.5856	244	3.8126	0.5478	—
20	6.63	—	—	—	1017	274	3.9774	0.6213	254	3.8675	0.5723	13.8
21	6.65	—	—	—	1017	246	3.7387	0.5937	252	3.8353	0.5776	2.0
22	6.65	—	—	—	1018	254	4.0008	0.6625	252	3.8684	0.5946	1.5

TABLE XIV.
METABOLISM OF SULFUR IN SULFATE.

PERIOD FIGURES.

Occurrence.	First Period, Days 1-5.			Second Period, Days 6-17.			Third Period, Days 18-22.		
	S in Total SO ₄	S in Combined SO ₄	S in Pre-formed SO ₄	S in Total SO ₄	S in Combined SO ₄	S in Pre-formed SO ₄	S in Total SO ₄	S in Combined SO ₄	S in Pre-formed SO ₄
Urine	grams 0.6276	grams 0.0762	grams 0.5514	grams 1.7005	grams 0.1806	grams 1.5199	grams 0.9745	grams 0.1074	grams 0.8671
Feces	0.0193	—	—	0.0984	—	—	0.0024	—	—
Cage washings	0.0439	—	—	0.0511	—	—	0.0301	—	—
Total	0.6908	—	—	1.8500	—	—	1.0160	—	—

DAILY AVERAGES.

Urine	0.1255	0.0152	0.1103	0.1417	0.0151	0.1266	0.1940	0.0215	0.1734
Feces	0.0039	—	—	0.0082	—	—	0.0005	—	—
Cage washings	0.0086	—	—	0.0043	—	—	0.0078	—	—
Total	0.1380	—	—	0.1542	—	—	0.2032	—	—

METABOLISM OF PHOSPHORUS IN PHOSPHATE.

	P in PO ₄ Period Figures.	P in PO ₄ Daily Averages.	P in PO ₄ Period Figures.	P in PO ₄ Daily Averages.	P in PO ₄ Period Figures.	P in PO ₄ Daily Averages.
Urine	0.4031	0.0806	1.2208	0.1017	0.5591	0.1118
Feces	1.1494	0.2299	5.5089	0.4591	0.8033	0.1607
Cage washings	0.0106	0.0021	0.0133	0.0011	0.0100	0.0020
Total	1.5631	0.3126	6.7430	0.5619	1.3724	0.2745

TABLE XV.

ANALYTIC TOTALS AND AVERAGES.

Elements.	Nitrogen.			Sulfur.			Phosphorus.		
	I. 5 days.	II. 12 days.	III. 5 days.	I. 5 days.	II. 12 days.	III. 5 days.	I. 5 days.	II. 12 days.	III. 5 days.
Food	grams 22.4585	grams 53.9004	grams 22.4585	grams 1.4160	grams 3.3984	grams 1.4160	grams 6.1110	grams 14.6664	grams 6.1110
Excretions	18.4883	47.9899	20.5620	1.2546	3.0685 (3.2186)*	1.3784	3.2982	13.4497	2.9460
Balance	+3.9702	+5.9105	+1.8965	+0.1614	+0.3200 (+0.1708)	+0.0376	+2.8128	+1.2167	+3.1650

AVERAGE DAILY BALANCES.

	+0.7940	+0.4925	+0.3793	+0.0323	+0.0275 (+0.0150)	+0.0075	+0.5626	+0.1014	+0.6330
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TOTAL AMOUNTS IN EXCRETIONS.

Urine	16.4300	42.5548	19.3420	0.8440	2.1334	1.1712	0.8119	2.4488	1.1354
Cage washings	0.3753†	0.7868	0.5961	0.0727	0.0943	0.0712	0.0274	0.0700	0.0433
Feces	0.6981	3.3137	0.3609	0.1090	0.5712 (0.7213)	0.0709	2.3918	10.8396	1.7507
Hair	0.0759	1.3346	0.2630	0.2280	0.2606	0.0561	0.0671	0.0013	0.0176
Total	18.4883	47.9899	20.5620	1.2546	3.0685 (3.2186)	1.3784	3.2982	13.4497	2.9460

* The figures in parentheses are explained on page 403.

† About one-fourth of the cage washings was accidentally spilled; most of this was recovered.

TABLE XV.—CONTINUED.

AVERAGE DAILY AMOUNTS IN EXCRETIONS.

Urine	3.2878	3.5462	3.8684	0.1690	0.1778	0.2342	0.1624	0.2041	0.2271
Cage washings	0.0750	0.0656	0.1192	0.0145	0.0079	0.0142	0.0055	0.0060	0.0035
Feces	0.1396	0.2761	0.0722	0.0218	0.0476 (0.0601)	0.0160	0.4784	0.9033	0.3501
Hair	0.1952	0.1112	0.0526	0.0456	0.0225	0.0112	0.0134	0.0076	0.0035
Total	3.6976	3.9001	4.1124	0.2500	0.2558 (0.2683)	0.2756	0.6597	1.1210	0.5897

DAILY AMOUNTS IN FOOD.

	4.4917	0.2832	1.2222
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TABLE XVI.

QUANTITIES OF FECES, CAST-OFF HAIR AND SCURF FOR EACH PERIOD.

Periods.	I 5 days.	II 12 days.	III 5 days.
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FECES

Total weight, dry	grams 25.5	grams 123.1	grams 17.8
Average daily weight, dry	5.1	10.26	3.56

CAST-OFF HAIR AND SCURF.

Total weight	7.4	10.1	1.95
Average daily weight	1.48	0.84	0.39

Nitrogenous Catabolism.—The figures in Tables XIII and XV seem to show that there was a stimulation of catabolic processes. We believe, however, that there was a uniformly decreasing tendency, from the beginning of the experiment, to store nitrogen, and that this tendency was not sufficiently influenced by the administration of the radium to warrant a positive conclusion in favor of accelerated catabolism. The slightly increased elimination of urine, in each successive period, which can hardly be attributed to the influence of either the radium or to the barium accompanying it, probably explains mechanically the decreased retention of nitrogenous products in the second and third periods of this experiment.

Sulfur Catabolism.—The results for sulfur are parallel with those for nitrogen. It may be presumed, in harmony with the suggestions on page 389, that the figures for sulfur in feces actually obtained are low by the amount of sulfur, 0.1501 gram, presumably precipitated as radium and barium sulfates by the 1.3916 grams of bromid fed to the dog. The figures for sulfur in Table XV which are not in parentheses are those actually obtained. The figures in parentheses are the actual figures corrected for the sulfur precipitated by the bromid. In calculating this quantity, the molecular weight of the radium bromid (containing barium bromid) was assumed to be the same as that of barium bromid, *i. e.*, 297.32. The small quantity of radium present (see p. 373) makes an inappreciable difference in the calculated result for the weight of precipitated sulfur.

Sulfate-Sulfur Catabolism.—The results for sulfate sulfur, shown in Table XIV, are parallel with those for nitrogen.

Phosphorus Catabolism.—There was an increased elimination of phosphorus during the second period, due to the increased daily quantity of feces, as is shown in Table XVI. That the increased phosphorus elimination was registered in the feces is shown clearly in Tables XIV and XV. Although, as in Experiment I, the barium evidently stimulated intestinal peristalsis, there was practically no diarrhoea. The figures for phosphorus in the urine are entirely parallel with those for nitrogen and sulfur, a fact in harmony with our conclusions that the variation in phosphorus excretion was due to increased fecal elimination, rather than to a real, increased phosphorus catabolism.

Phosphate-Phosphorus Catabolism.—The results for phosphate-phosphorus, shown in Table XIV, are parallel with those for phosphorus, described above.

IX. FOURTH METABOLISM EXPERIMENT. INFLUENCE OF RADIUM BROMID (CONTAINING BARIUM BROMID), INJECTED SUBCUTANEOUSLY.

Diet.—The character of the diet was the same throughout the entire experiment.

TABLE XVII.

COMPOSITION OF THE DAILY DIET.

Ingredients.	Hashed Beef.	Cracker- Meal.	Lard.	Bone-ash.	Water.	Total.
Weight.	grams 100.0	grams 20.0	grams 10.0	grams 5.0	grams 225.0	grams 360.0
Nitrogen	3.5460	0.3216	0.0006	0.0002	—	3.8684
Sulfur	0.2001	0.0263	0.0001	0.0075	—	0.2340
Phosphorus	0.2239	0.0420	0.0010	0.9051	—	1.1720

Preparatory Period.—A preparatory period of six days was sufficient to get the dog accustomed to his surroundings, and into approximate nitrogenous equilibrium. At 10 A.M., June 13, 1905, the collection of material for analytical work was begun.

First Period.—*Normal condition.*—*Maintenance of approximate nitrogenous equilibrium.* Days 1-7, June 14-20, 1905. The animal was further accustomed to its environment.

Second Period.—*Metabolic influence of radium bromid, 10,000 activity (containing barium bromid), injected subcutaneously.* Days 8-15, June 21-28, 1905. At 4.08 P. M., June 20, 1905, the dog was given the first injection. Two more injections were given, one on each of the two following days. The amounts injected were 0.0173, 0.0217, and 0.0372 gram, for the first, second, and third injections, and are very nearly 3, 4, and 7 mg. per kilo respectively. The after effects of the three injections were looked for in the five days following. This was, therefore, both an experimental and an after-period. The dog remained in the cage, under observation, until 10 A.M., July 12, 1905. During this period nothing abnormal in the appearance or the behavior of the dog was observed.

Analytical Results.—The analytical data for the fourth experiment are given in Tables XVII-XXI.

TABLE XVIII.

DAILY RECORDS OF THE FOURTH METABOLISM EXPERIMENT.

First period—Maintenance of normal approximate nitrogenous equilibrium, June 14-20, 1905.

Day No	Body Weight Kilos.	Radium Bromid 10,000 Activity.		Urine.						Feces.
		Total Dose.	Mg. per Kilo. Body Weight	Volume.	Specific Gravity.	Nitrogen.	Total daily Sulfate.	Period Average to Date.		Weight Dry.
								Volume.	Nitrogen.	
		grams		cc.		grams	grams	cc	grams	grams
1	5.68	—	—	196	1018	3.4505	0.3843	196	3.4505	0.3843
2	5.68	—	—	180	1016	3.0868	0.3961	188	3.2686	0.3902
3	5.67	—	—	203	1020	4.1763	0.5734	193	3.5712	0.4513
4	5.74	—	—	139	1015	2.4761	0.2861	179	3.3974	0.4100
5	5.71	—	—	210	1021	4.4975	0.6707	186	3.5374	0.4621
6	5.71	—	—	193	1014	2.7370	0.3294	187	3.4040	0.4400
7	5.66	—	—	232	1019	3.8120	0.5268	193	3.4623	0.4524

Second period—Metabolic influence of radium bromid injected subcutaneously, June 21-28, 1905.

8	5.56	0.0173	3	278	1014	3.1552	0.4019	278	3.1552	0.4019	18.0
9	5.56	0.0217	4	238	1014	3.1641	0.3754	258	3.1596	0.3886	—
10	5.52	0.0372	7	257	1016	3.7794	0.4819	258	3.3662	0.4148	9.0
11	5.49	—	—	228	1018	3.9335	0.4020	250	3.5080	0.4153	6.5
12	5.52	—	—	200	1015	3.0502	0.4031	240	3.4165	0.4129	8.0
13	5.52	—	—	216	1015	3.4820	0.4626	236	3.4274	0.4212	8.3
14	5.58	—	—	148	1014	2.0421	0.2711	224	3.2295	0.3997	8.3
15	5.57	—	—	252	1018	4.4511	0.6077	227	3.3822	0.4257	7.8
29	5.47	—	—	—	—	—	—	215	—	—	—

TABLE XIX.

METABOLISM OF SULFUR IN SULFATE.

PERIOD FIGURES.

Occurrence.	First Period, Days 1-7.			Second Period, Days 8-15.		
	S in Total SO ₄ .	S in Combined SO ₄ .	S in Preformed SO ₄ .	S in Total SO ₄ .	S in Combined SO ₄ .	S in Preformed SO ₄ .
	grams	grams	grams	grams	grams	grams
Urine	1.0881	0.0619	1.0262	1.1487	0.0691	1.0796
Feces	0.0425	—	—	0.0429	—	—
Cage washings	0.0183	—	—	0.0145	—	—
Total	1.1489			1.2061		

DAILY AVERAGES.

Urine	0.1554	0.0088	0.1466	0.1436	0.0086	0.1350
Feces	0.0061	—	—	0.0054	—	—
Cage washings	0.0026	—	—	0.0018	—	—
Total	0.1641			0.1508		

METABOLISM OF PHOSPHORUS IN PHOSPHATE.

	P in PO ₄ Period Figures.	P in PO ₄ Daily Averages.	P in PO ₄ Period Figures.	P in PO ₄ Daily Averages.
Urine	0.7894	0.1128	0.8126	0.1016
Feces	2.8796	0.4114	3.0776	0.3847
Cage washings	0.0053	0.0008	0.0047	0.0006
Total	3.6743	0.5250	3.8949	0.4869

TABLE XX.

ANALYTIC TOTALS AND AVERAGES.

Elements.	Nitrogen.		Sulfur.		Phosphorus.	
	I 7 days.	II 8 days.	I 7 days.	II 8 days.	I 7 days.	II 8 days.
	grams	grams	grams	grams	grams	grams
Food	27.0788	30.9472	1.6380	1.8720	8.2040	9.3760
Excretions	26.2714	29.3115	1.7186	1.9634	7.7423	8.2487
Balance	+0.8074	+1.6357	-0.0806	-0.0914	+0.4617	+1.1273

AVERAGE DAILY BALANCES.

+0.1153	+0.2044	-0.0115	-0.0114	+0.0660	+0.1409
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TABLE XX.—CONTINUED.

TOTAL AMOUNTS IN EXCRETIONS.

Urine	24.2362	27.0576	1.2887	1.4786	1.5932	1.5680
Cage washings	0.1378	0.1426	0.0291	0.0326	0.0104	0.0145
Feces	1.3506	1.5840	0.2810	0.3374	6.1012	6.6492
Hair	0.5468	0.5273	0.1198	0.1148	0.0375	0.0170
Total	26.2714	29.3115	1.7186	1.9634	7.7423	8.2487

AVERAGE DAILY AMOUNTS IN EXCRETIONS.

Urine	3.4623	3.3822	0.1841	0.1848	0.2276	0.1960
Cage washings	0.0107	0.0178	0.0042	0.0041	0.0015	0.0018
Feces	0.1929	0.1980	0.0401	0.0422	0.8716	0.8311
Hair	0.0781	0.0659	0.0171	0.0143	0.0055	0.0021
Total	3.7530	3.6639	0.2455	0.2454	1.1062	1.0310

DAILY AMOUNTS IN FOOD.

	3.8684	0.2340	1.1720
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TABLE XXI.

QUANTITIES OF FECES, CAST-OFF HAIR AND SCURF FOR EACH PERIOD.

Periods.	I 7 days.	II 8 days.
FECES.		
Total weight, dry.	grams. 59.2	grams. 66.5
Average daily weight, dry.	8.4	8.3
CAST-OFF HAIR AND SCURF.		
Total weight	5.2	5.0
Average daily weight	0.74	0.62

Discussion of Results.—The 0.0762 gram of 10,000 activity radium bromid had very little effect on the weight of the dog. The increased elimination of urine during the second period was probably due to the dog's nervous excitement at the time when the injections were made. The fall in the specific gravity of the urine during the injection days harmonizes with this view. This is also the probable cause of the decreased weight in the second period. No unusual variations in the quantity or specific gravity of the urine, or in the consistency and quantity of feces and epidermal waste matter, were observed. All of the

feces eliminated after the radium bromid injections, and up to July 12, 1905, were radio-active (see Discussion of results, and footnote thereto, pp. 399 and 402). The blood, spleen, pancreas, and gastrocnemius muscle were not radio-active. The stomach, lungs, kidneys, brain, and muscles near the place of injection were slightly radio-active. The liver was more radio-active than any other of these parts.

*Nitrogenous Catabolism.*¹—The figures in Tables XVIII and XX indicate that there was a slight inhibition of catabolic processes. The average daily amount of nitrogen in the urine during the second period was lower than during the first, in spite of the increased daily volume of urine eliminated during the second period. At the same time, these results are not decided enough to warrant any very definite conclusions, one way or the other.

Sulfur Catabolism.—This was not affected. The quantity of

¹ The following table gives the results of the analyses of the composite urines, which were made as a check on the daily results. Total nitrogen in the daily urine was determined in duplicate, the recorded daily amount being the averages of two very close results. In some cases, the determination of nitrogen in the composite samples was made nearly three months after that in the daily urine, and, as the table shows, the thymol added to the urine acted as an efficient preservative.* The differences between the amounts of total nitrogen in the daily and the composite samples are entirely within the limits of error for such determinations, and show that the total nitrogen content of urine so preserved, remains constant.

Experiment. No.	Period No.	Number of Days in Period.	Composite Urines.	Total Nitrogen, in grams.		
				Daily Samples.	Differences.	
					Total.	Daily.
1	1	9	34.6360	34.6940	0.0580	0.0064
	2	19	68.6000	68.9165	0.3165	0.0167
2	1	7	31.2480	31.2869	0.0389	0.0056
	2	12	50.6000†	51.2618	0.6618	0.0551
3	1	5	16.1780	16.4390	0.2610	0.0522
	2	12	42.1270	42.5548	0.4278	0.0356
	3	5	19.2820	19.3420	0.0600	0.0120
4	1	7	24.4575	24.2362	0.2213	0.0316
	2	8	27.0810	27.0576	0.0234	0.0029

* Mead and Gies, *loc. cit.*, p. 107.

† Difficult to sample, on account of small amount of fecal matter present.

sulfur, about 0.007 gram, probably precipitated as sulfate by the injected radium bromid is too small to need consideration here.

Sulfate-Sulfur Catabolism.—This was not affected.

Phosphorus, and Phosphate-Phosphorus Catabolism.—The results for total phosphorus and for phosphate-phosphorus are practically the same as those for nitrogen.

X.—GENERAL REMARKS ON THE RADIUM METABOLISM EXPERIMENTS.

In Metabolism Experiments III and IV, it was evident that no definite metabolic effects were observed that could be attributed to radium. Protein catabolism appeared to be very slightly *accelerated* in the third experiment. It seemed to be *inhibited* to about the same degree in the fourth. In neither experiment were the metabolic balances great enough to make it certain that they were not due to the many and unavoidable sources of variation commonly recognized in such work. It is probable, of course, that larger doses of more active radium preparations would have given definite results, but that the whole system of the animal in each of these two experiments was permeated by radio-active matter, however slight in proportion, was shown by the radio-activity of the fecal discharges in the fourth experiment (p. 408). The prohibitive cost of radium preparations of higher activity made further experimentation in this direction impossible.

XI.—SUMMARY OF GENERAL CONCLUSIONS.

In control experiments on the toxicity of barium bromid numerous observations of previous investigators were confirmed.

Radium preparations of low activity (240, 1,000, 10,000) containing barium bromid in predominating proportions were without special influence on metabolism when administered per os or subcutaneously in relatively large quantities. Equal or larger doses of pure barium bromid also failed to show any decisive metabolic effects before fatal results were inaugurated.

After subcutaneous injection, radium (bromid), like barium, calcium, and similar elements, is eliminated per rectum. Elimination occurs speedily. The intestine seems to be the main channel of radium excretion.

Radium bromid, whether introduced per os or subcutaneously, appears to be eliminated in slight amounts in the urine. It does not appear to be eliminated through the skin or in epidermal matter.¹

The experiments herein described have been carried out under the constant care and supervision of Dr. William J. Gies. We take pleasure in acknowledging our indebtedness to him for the thorough instruction, and ever-ready willingness to assist us by word and deed, in this work.

NOTES ON THE REMARKS MADE BY DR. HAWK REGARDING THE
CAGE AND FEEDING METHODS USED IN THE EXPERI-
MENTS DESCRIBED IN THE FOREGOING PAPER.

Shortly after the proofs of the foregoing paper had been corrected, there was brought to our attention a reprint of Hawk's recent contribution in the *University of Pennsylvania Medical Bulletin* for December, '05, entitled "Description of a Convenient Form of Cage for Use in Metabolism Experiments on Dogs, and of a New Method for the Preservation of Meat Used in Such Experiments." In all of our work with barium and radium we used the cage and the feeding methods that were described by Gies² and which were referred to by Hawk unfavorably, in some respects, in the aforesaid contribution.

With regard to the method of preserving meat for metabolism experiments, which has been steadily in use in this laboratory for over five years, Hawk says: "The only feature of the process which seemed to us unsatisfactory was the custom of making the meat into balls and keeping them in small glass receptacles. Of course, very shortly after the meat has been placed in the cold storage it will freeze solid, and while *it is a very simple matter to remove any number of the small balls intact from their receptacle*,³ it is somewhat more difficult to cut off any desired amount from one of the frozen balls *while it is still within the glass vessel*." This process takes an appreciable length of time and during this time the contents of the jar are in contact with the air. The temperature of the ordinary working room, where the weights of the various constituents of the diet are usually made, being so much above that of the cold storage room, it takes a very short time for the frozen meat to show signs of thawing, and this of itself may introduce an error. It also occasionally happens, that because of the difficulty in cutting the frozen meat *while in the receptacle*³ or because of the desire of the experimenter to

¹ An interesting research, by Elster and Geitel, on the elimination of radio-active material through the lungs, is reviewed in the *Archives of the Roentgen Ray*, p. 178, January, 1905.

² Gies, *loc. cit.*

³ Italics our own.

hasten the process, the receptacle is broken or cracked and the meat, in excess of what is needed for that particular meal, must be discarded."

Why, it may be asked, should one struggle to cut off any desired amount of meat from one of the frozen balls, "*while it is still within the glass vessel,*" when the balls may be "*so easily removed from the container,*" and when, by a simple shaving process with a scalpel, any necessary quantity of meat may be quickly obtained from the last ball involved in the process, as the original description of the method recommended. It appears to us that Hawk has criticised, not the method that we have used in our work with perfect satisfaction, but some one's perversion of that method. Hawk's proposal to use paraffin paper coverings to prevent the imaginary defects and errors alluded to is answered by the fact that repeated analyses of meat, which was properly preserved by the method we used and exposed to room temperature for its removal from the containers (during the very short intervals that were necessary for the *comfort* of the operator), have shown that the meat does not undergo any change of composition that can be detected by analysis.

In referring to the style of cage which has been in use in this laboratory for about three years, Hawk states the following: "In our experiment with cages similar to this type, we have found them defective in but a single particular, *i.e.*, in the device for the collection of the urine. . . . It has, therefore, been our desire to secure a cage, which, while comparatively cheap, should possess all the good qualities of the type of cage mentioned above and at the same time be provided with an improved device for the collection of the urine."

That Hawk has adopted for his cage only a few of the many advantages alluded to will be apparent to any reader of the recent description of our cages or to any one who uses them. With regard to the "defect" of the cage used by us and others, Hawk says: "It was our experience with the old method of collecting urine, by means of a bottle, which was not permanently fastened to the movable drip pan, that there was always danger of forgetting to remove the urine receptacle before drawing the pan, and in this way *the risk was encountered of overturning the receptacle and suffering the loss of a valued specimen of urine.*¹ By means of the device described above, however, there is no possibility of the loss of a urine sample in this way. With this device, whenever the sliding pan is drawn forward, the urine receptacle is likewise moved forward, and its position in relation to the sliding pan is never altered."

Hawk does not suggest that the same forgetfulness that might be responsible for "overturning the receptacle and suffering the loss of a valued specimen of urine" in the manner indicated would probably also be responsible for the same result from damage to the forgotten receiver by striking it against the floor, the side of a table, and so on, in thoughtlessly handling the drip pan.

¹ Italics our own.

The criticism just quoted has been offered by Hawk in spite of the following statements in Gies's description of his cage regarding the urine receiver: ". . . If the height of the bottle and funnel, when the latter is in place, is very slightly less than the distance from the shelf to the lower edge of the outlet tube from the drip pan, either the drip pan or the shelf may be moved in and out without bringing the outlet tube of the drip pan in contact with the funnel of the receiver, when the latter is in the accustomed position on the shelf. *Under these circumstances it is impossible to upset the receiver, if the drip pan is pulled out before the receiver has been moved away from its usual position.*¹ . . . " Urine receivers of this character have given us perfectly satisfactory results.

Gies also stated that "the drip pan is so arranged that numerous modifications in the method of collecting the urine are possible." This remark makes general allusion to several modifications that the workers in this laboratory have repeatedly discussed during the past half-dozen years, each of which the cage permits according to individual preferences or needs. Hawk has expressed the idea that "in practically every form of cage which is used in metabolism work upon the lower animals at the present time, the device for the collection of the urine is not entirely satisfactory." Even in cages provided with a movable drip pan, he says, there is no other device for the collection of the urine, so far as he is aware, "which is fastened to the pan in such a manner as to move with the movement of the pan." A simpler and more convenient device than the one described by Hawk, attached to cages of this general design, has been in use for the past two years in the Laboratory of Pharmacology at this College.

¹ Italics our own

GLYCOCOLL PICRATE.

By P. A. LEVENE.

(From the Rockefeller Institute for Medical Research)

(Received for publication, January 16, 1906.)

The separation of glycocoll from a mixture of amido-acids and its identification were greatly facilitated by the method of E. Fischer. The method depends on the transformation of the substance into the hydrochloride of its ethylester. However, in mixtures in which the quantity of glycocoll does not largely exceed that of other acids, the separation offers great difficulties.

In the course of a study of the products of tryptic digestion of gelatin, the writer has made the observation that glycocoll can be separated and analyzed in form of its picrate.

It is possible to obtain from mixtures of the products of hydrolytic cleavage of proteids fractions consisting of glycocoll and alanin. From such mixtures glycocoll can be isolated in form of its picrate. This is obtained in the following manner: To one part of glycocoll dissolved in very little hot water, four parts (by weight) of picric acid dissolved in alcohol are added. On cooling, the picrate comes down in form of light yellow shining scales. They are readily recrystallized out of water. Dried in vacuo over sulphuric acid, the substance has a melting-point of 190° C.

Sample I was prepared from a mixture of alanin and glycocoll obtained from the products of digestion of gelatin by means of trypsin. The mixture had the following composition:

0.1122 gr. of the substance gave on combustion 0.1593 gr. CO_2 and 0.0790 gr. H_2O .

0.1187 gr. of the substance was employed for a Kjeldahl nitrogen estimation. It required for neutralization 14.25 c.c. $\frac{N}{10}$ H_2SO_4 .

	C	H	N
Calculated for $\text{C}_2\text{H}_5\text{NO}_2$, 32.00 per cent.	6.66 per cent.	18.66 per cent.	
" " $\text{C}_3\text{H}_7\text{NO}_2$, 40.44 "	7.85 "	15.75 "	
Found	38.71 "	7.83 "	16.81 "

Thus it is evident that the mixture contained more alanin than glycocoll.

The picrate obtained from it had the following composition:

0.1500 gr. of the substance gave on combustion 0.1720 gr. CO_2 and 0.0387 gr. H_2O .

For $\text{C}_2\text{H}_5\text{NO}_2 \cdot \text{C}_6\text{H}_3\text{N}_3\text{O}_7$

	<i>Calculated</i>	<i>Found</i>
C =	31.58 per cent.	31.86 per cent.
H =	2.63 " "	2.83 " "

Another preparation was obtained from a purer sample of glycocoll derived in the same manner as the mixture used for the previous experiment. The picrate had the following composition:

0.1920 gr. of the substance gave on combustion 0.2225 gr. of CO_2 , and 0.0514 gr. of H_2O .

For $\text{C}_2\text{H}_5\text{NO}_2 \cdot \text{C}_6\text{H}_3\text{N}_3\text{O}_7$

	<i>Calculated</i>	<i>Found</i>
C =	31.58 per cent.	31.60 per cent.
H =	2.63 " "	2.97 " "

ON GAS PRODUCTION BY FECAL BACTERIA GROWN ON SUGAR BOUILLON.

By C. A. HERTER AND HERBERT C. WARD.

(Received for publication, January 25, 1906.)

The ingenious and helpful studies by Professor Theobald Smith of pure cultures of bacteria grown in fermentation tubes suggested to us that something might be learned of the physiological attributes of the mixed bacteria of the human feces when these are grown in health or disease in the anaërobic portion of fermentation tubes. We desire to record here briefly the results of observations on the gas production by mixed fecal bacteria introduced (in a water-suspension designed to secure representative bacteria) into fermentation tubes containing sugar-peptone solutions. The observations on gas production were recorded at the end of about forty-eight hours' sojourn in the incubator at 37° C., and are given in terms of the height of the columns of gas in millimeters. Four sugar-peptone media were used.

The basis of the media was a bouillon medium to which were added different sugars in a concentration sufficient to give in each instance a concentration of two per cent. The sugars used were dextrose, a levulose-dextrose mixture (Schering's diabetin) lactose, and saccharose. Nearly uniform results in gas production were obtained in duplicate series when care was taken to insure a uniform distribution of the inoculated fecal bacteria.

The gas production by the mixed, unisolated fecal bacteria of normal individuals was found to be influenced somewhat by dietetic conditions and age. Presumably healthy, breast-fed children showed a smaller gas production than children who were bottle-fed or than children or adults on mixed diet. In the present communication reference will be made mainly to the gas production by the fecal bacteria of normal and pathological adults and of children who are no longer infants.

The average gas production noted in sixteen observations on the fecal bacteria of normal individuals was 103.65 mm. for the

four tubes. The largest gas production among these was 138, 137, and 129 mm., the smallest, 65, 69, and 72 mm.¹ As a rule, the largest gas production was in the lactose-peptone tube, the smallest, in the saccharose tube. The averages of gas production for the different individual tubes are as follows:

Dextrose	Levulose-dextrose	Lactose	Saccharose
26.75 mm.	27.5 mm.	29.9 mm.	19.5 mm.

The gas formed under the conditions that have been described was in some instances subjected to the absorption test by a caustic potash solution. The proportion of gas absorbed varied somewhat in the different sugar-peptone tubes. It may be said that, as a rule, from about one-quarter to one-tenth of the total gas (representing carbon dioxide) was absorbed. The proportion of carbon dioxide production was about the same in the cases where gas formation was inhibited.

Some observations have been made on the gas production by pure cultures of bacteria normally inhabiting the gastro-enteric tract. For example, one strain of *B. coli communis* gave 90 mm. of gas in the four tubes, while another strain gave 92 mm. *B. lactis aërogenes* gave 76 mm. All these values are somewhat below those obtained by growing the mixed fecal bacteria from presumably normal persons. Greater gas production was noted where *B. coli* was grown with *B. aërogenes capsulatus*.

The fact which we desire to bring forward in this communication is that there are pathological conditions in which the production of gas by the mixed fecal bacteria is distinctly less than is usual for normal persons. The feces from a number of persons showing the evidences of excessive intestinal putrefaction (putrefactive products in the feces or derivatives of putrefactive products in the urine) have been studied with respect to their gas production. It was found that a somewhat diminished gas production is a not infrequent accompaniment of various digestive disorders. A pronounced reduction in gas formation is less common, and when persistent is apparently associated with graver clinical manifestations.

Thus in a man of thirty-two years, suffering from pernicious anæmia, the gas production in the four tubes was only 30 mm.

¹ The anaërobic portion of the tubes used by us is about 9.5 cm. in length.

This was soon after entering the hospital. About a week later, during a diarrhoeal period the gas production rose to 100 mm. but declined again later to 60 mm.

In another patient with pernicious anæmia, the feces from a diarrhoeal stool gave 155 mm. of gas; some weeks later a formed stool gave bacteria which produced 45 mm. of gas. In a third patient with the same disease, a diarrhoeal stool gave 95 mm. of gas, but later the fecal bacteria from a formed stool gave 70 mm. only.

In another patient with pernicious anæmia, the semi-solid stool contained bacteria which gave 80 mm. of gas. As the patient improved, under the influence of rest and care in diet, the gas production increased to 90 mm. and later, coincidentally with further improvement, to 112 mm. Another patient with pernicious anæmia, a child of one year, gave on one occasion 35 mm. of gas, on another 38 mm.

Very low gas production was repeatedly observed in a severe case of diabetes on the verge of coma.

The smallest gas production has been noted in bottle-fed children suffering from marasmus.¹ In several instances the fecal bacteria from such children have failed to make a volume of gas measurable by ordinary methods. In one highly anæmic child in a marantic state the gas production amounted to 18 mm. when the first observation was made. After a week in bed, on a carefully chosen diet, the gas production was 105 mm. One week later it was 120 mm., and two weeks later 96 mm. The increase in gas production coincided with a striking improvement in nutrition and in the blood picture.

A marked fall in gas production has been noted during fever in a case where previous to the rise in temperature the gas production was large.

The explanation of the phenomena recorded here is not yet clear, but it appears likely that it is attributable at least in some instances to an interference with the normal gas-producing properties of organisms of the *B. coli communis* group. One may think of the gas-forming kinds of bacteria as actually dying out to a large extent in the lower bowel, or one may imagine them

¹ Our observations on bottle-fed children are not sufficiently numerous to enable us confidently to give the normal gas production for them. It appears to vary from 50 to 100 mm.

to be simply inhibited in their growth by the presence of bacteria which are limited gas-producers. The view that the gas-makers of the upper bowel are replaced in the lower bowel very largely by organisms with other physiological character is substantiated in a noteworthy manner by the results of studies with the gram stain. Bacteria of the *B. coli* group are gram negative, and where we have preponderantly gram-negative stools containing, *B. coli* in large numbers one would expect an average gas production by fecal bacteria grown on sugar bouillon. This has been actually the case. On the other hand, gram-positive feces containing bacteria of the *B. coli* type are small gas-producers. A special instance of a physiological flora which is able to make little gas and is gram-positive is that of the breast-fed infant in which *B. coli* has not become established and in which *B. bifidus* (Tissier) and *B. acidophilis* (Moro) are dominant. In adults and in children on cow's milk a gram-positive stool is usually not physiological, and in such cases it may be that small gas production is partly dependent on the inhibiting action of some pseudo-parasitic organism or combination of organisms.

We have repeatedly observed a change in the fecal flora from a gram-positive to a gram-negative character and with this change an increase in gas production. In an autopsy on a child dead of pneumonia, the following conditions were observed with respect to gas production. Bacteria from the stomach gave 40 mm. of gas; bacteria from the duodenum, 94 mm. of gas; cultures from the jejunum gave 78 mm. of gas, and the same amount was obtained from the cultures from the ileum; and finally bacteria from the rectum gave only 22 mm. of gas.¹ In the rectum the bacteria were gram-positive; above the rectum, mainly gram-negative.

We have not yet been able to reproduce experimentally the impaired gas formation by combining with the colon bacillus some organism capable of inhibiting its gas-producing qualities. A definite association between gas production by the fecal bacteria under anaërobic conditions of growth and special conditions of intestinal putrefaction has not been established.

¹ This observation harmonizes with the fact that the diarrhoeal flora of the human intestinal contents generally give more gas than the bacteria derived from formed movements from the same individual.

We believe that further studies of the phenomena here described will prove to be of biological interest and of value in clinical investigations of intestinal disorders.

Note.—A great increase in gas production was noticed in a monkey which developed diarrhœa after feeding with cabbage. This observation is in accord with what we have noticed in connection with human diarrhœa.

THE PRODUCTION OF METHYL MERCAPTAN BY FECAL BACTERIA GROWN ON A PEPTONE MEDIUM.

By C. A. HERTER.

(Received for publication, January, 22, 1906.)

It is surprising that almost no systematic study has been devoted to methyl mercaptan, a common product of the putrefactive cleavage of proteid, in its relation to intestinal putrefaction. Nencki,¹ who discovered this substance among the products of putrefaction, obtained a small amount of it from a large quantity of human feces, and reached the conclusion that it is a usual product of intestinal putrefaction. This conclusion is probably correct, although, strictly speaking, it does not appear fully justified by Nencki's observations, for the reason that the material used by him could not have been sufficiently fresh to exclude mercaptan formation during a period of exposure to the air. Working with smaller quantities of material than were employed by Nencki, I have never been able to obtain more than a trace of mercaptan (probably the methyl compound), and have usually been able to obtain no evidence whatever of its presence in fresh human material, whether from presumably normal persons or from such as were suffering from intestinal disturbance. But the failure to find mercaptan in the contents of the lower bowel does not prove that this substance has not been formed, for it is possible and even likely that there is some mercaptan production in higher portions of the large intestine, where it is certain that the gas would be readily absorbed.

As it appeared that something of interest might be learned from the study of the ability of fecal bacteria from various sources to form mercaptan when growing on a medium which does not readily yield this compound, a considerable number of experiments were made with suspensions of the mixed fecal bacteria from different persons. After growing the bacteria in a two per cent. peptone solution for twenty-four hours at the temperature of

¹ *Monatsh. f. Chem.*, x, p. 862, 1889. Das Methylmercaptan als Bestandtheil der menschlichen Darmgase.

37° C., the entire culture (100 c. c.) was in each case transferred to a flask communicating through a calcium chloride tube with an Erlenmeyer flask containing isatin dissolved in concentrated sulphuric acid. A current of air was then drawn through both flasks so that any mercaptan given off from the culture flask would enter the isatin-sulphuric-acid flask. The presence of mercaptan is indicated under these conditions by a gradual change of the isatin solution from red to olive-green or grass-green.¹ The method is not adapted for quantitative determinations but some idea can be gained through it of the quantity of mercaptan present in a culture, and it further serves to indicate differences in the amount formed in different cultures. Twenty-five milligrams of a one per cent. solution of methyl mercaptan suffice to gradually alter the red isatin solution (about 50 c. c.) to a deep green in the course of ten minutes. Reactions as strong as this are occasionally obtained from one hundred cubic centimeters of a bacterial culture, but quicker reactions of this intensity have not been found.

With the aid of the method² here outlined, more than one hundred and thirty observations have now been made on aërobic cultures of mixed fecal bacteria.³ A fact which stands out clearly as a result of these observations is that the bacteria derived from normal persons (*i. e.*, showing not more than moderate quantities of putrefactive derivatives in the urine and otherwise in good health) do not usually yield more than a trace of mercaptan when grown for twenty-four hours on a two per cent. peptone solution. In a number of instances, a pronounced trace of mercaptan has been obtained after prolonged aspiration through the apparatus mentioned above. In a few instances, a moderate reaction has been obtained after aspiration for ten or fifteen minutes, and such a reaction

¹ This method has been used by Niemann, *Arch. f. Hyg.*, xix, p. 126, 1893; also by Bauer, *Zeitschr. f. physiol. Chem.*, xxxv, p. 346, 1902.

² The method was in many instances supplemented by the use of the mercuric cyanide method.

³ The organisms were grown in 250 c. c. flasks and the volume of the peptone solution was 100 c. c. The upper portion of the culture was under aërobic conditions, the lower part, under anaërobic conditions.

has sometimes been repeatedly obtained from bacteria from the same person. Strong reactions were several times obtained from the organisms obtained from breast-fed babies apparently in good health, and once a strong mercaptan reaction was noted in the case of a growth made from material from a young man in good health but troubled with constipation. With these exceptions, I have noted strong mercaptan production only in cultures made from material derived from pathological sources. The strongest reactions were obtained from bacteria derived from persons suffering from pernicious anæmia, depressive mental states, infantile marasmus, fat diarrhœa, and cases of chronic intestinal indigestion (in children) characterized by abdominal distension, anæmia, and retarded development. In the cases referred to, mercaptan production has usually been a persistent rather than a transitory manifestation. In two cases of pernicious anæmia in which rapid improvement occurred in association with rest in bed and care in diet, the fecal bacteria ceased to produce mercaptan, coincidently with this improvement.

Thus the facts at present at our disposal indicate that the pronounced formation of methyl mercaptan by fecal bacteria growing on peptone solution is commonly a manifestation of pathological rather than normal bacterial activity, although it is doubtful whether its occasional production by micro-organisms from a human individual is to be regarded as necessarily unphysiological. Especially in the case of young children in good health is a moderate mercaptan a common occurrence. This conclusion is the more noteworthy because it is entirely different from the conclusion reached from a study of hydrogen sulphide production under similar condition. In the case of hydrogen sulphide formation, it may be said that this sulphur compound has almost regularly been obtained by growing fecal bacteria on a peptone medium, and that it is by no means uncommon to find an abundant hydrogen sulphide production where no trace of mercaptan is obtainable.¹

We have as yet little accurate information as to the conditions under which methyl mercaptan is produced in the course

¹ The bacteria from certain acid stools from children may fail to make hydrogen sulphide, although under anaërobic conditions mercaptan may be produced.

of bacterial activity. Nencki regarded it as a result of anaërobic decomposition, but it appears that, although this view is for the most part correct, anaërobic conditions are not always essential to its formation. The bacteria from the feces of a patient with pernicious anæmia grown on a peptone solution under aërobic conditions gave much hydrogen sulphide and no mercaptan; when the organisms were grown anaërobically (under carbon dioxide) there was an abundant production of mercaptan (presumably the methyl compound) in addition to hydrogen sulphide. On a medium containing asparagin, ammonium lactate, alanin, glycocoll, cystin, and salts, I obtained a very abundant production of hydrogen sulphide from fecal bacteria, but not a trace of mercaptan.

Pure cultures of the aërobic bacteria derived from feces capable of inducing mercaptan formation have failed to give this gas. *B. coli communis* growing on the peptone solution gives hydrogen sulphide but no mercaptan. *B. putrificus* I have not yet tried in pure culture, but Dr. Rettger tells me that it early produces mercaptan when grown on an egg-meat medium.

Although it is not at present clear what inferences may be safely made, from the formation of mercaptan by the fecal bacteria, with regard to mercaptan production and absorption in the intestine¹ from which the bacteria were derived, it seems clear that further observations are desirable along lines suggested by the results embodied in this paper. It appears also desirable that a careful pharmacological study of the prolonged action of methyl mercaptan should be undertaken, and such a study is now under way in my laboratory.

¹ Some observations which I have made upon bacteria found at early autopsies in various parts of the intestinal tract show that mercaptan-producing organisms may be present in the upper part of the ileum. Thus from an autopsy on a child of fifteen months, dead of pneumonia, the mercaptan production by the bacteria of the ileum was even more pronounced than that of bacteria obtained from lower levels. In the case of a child dead of marasmus, the stomach contained bacteria which made hydrogen sulphide abundantly and some mercaptan. The bacteria of the duodenum and jejunum made neither of these gases, but those of the ileum produced them both in abundance.

ON THE PYRIMIDIN BASES OF THE NUCLEIC ACID OBTAINED FROM FISH EGGS.

BY J. A. MANDEL AND P. A. LEVENE.

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(Received for publication, March 6, 1906.)

It is generally accepted that the unfertilized egg contains no true nuclein derivatives. This view is based on the work of A. Kossel, who failed to demonstrate the presence of purin bases in the unfertilized egg of the hen.

In the present work an attempt was made to obtain a true nucleic acid from fish eggs. The process employed in this study was the one by which Levene, and later the writers, obtained nucleic acid from various tissues.¹

A substance was obtained presenting the general properties of a true nucleic acid, except that in composition this acid was similar to the nucleic acid obtained from plant cells, which contains, as is well known, only two pyrimidin bases, viz., uracil and cytosin, while those of animal tissues and of spermatozoa contain besides these also thymin, which is usually present in larger quantities than the other bases.

The acid obtained from fish eggs yielded uracil instead of thymin, as was to be expected. In solubility and in its high content of reducing substance, the acid from fish eggs resembles the nucleic acids of plant cells.

On analysis, the copper salt of the acid gave 13.31 per cent. of nitrogen and 7.6 per cent. of phosphorus. The pyrimidin bases were separated from the acid by the process previously described by us.²

Thirty-eight grams of the air-dried copper salt were employed

¹ Levene, *Zeitschr. f. physiol. Chem.*, xlv, p. 370, 1905; Mandel and Levene, *ibid.*, xlvi, p. 155, 1905.

² Mandel and Levene, *loc. cit.*

for the analysis and one gram of uracil was obtained. Reanalysis of this substance gave the following results:

0.1308 gram of the substance yielded on combustion 0.2077 gram CO_2 and 0.0462 gram H_2O .

On the basis of the formula $\text{C}_4\text{H}_4\text{N}_2\text{O}_2$ the results are as follows:

<i>Calculated.</i>	<i>Found.</i>
C = 42.82 per cent.	43.30 per cent.
H = 3.59 per cent.	3.91 per cent.

Cytosin was obtained as a picrate which was transformed into the sulphate and chlorplatinate. The last served for analysis as follows:

0.1173 gram of the substance contained 0.0335 gram Pt. For the formula $(\text{C}_4\text{H}_5\text{N}_3\text{O})_2\text{PtCl}_4 \cdot 2\text{HCl}$ the quantities were the following:

<i>Calculated.</i>	<i>Found.</i>
Pt = 30.84 per cent.	30.10 per cent.

THE STIMULATING AND INHIBITORY EFFECTS OF MAGNESIUM AND CALCIUM UPON THE RHYTHMICAL CONTRACTIONS OF A JELLYFISH (POLYORCHIS).

By JACQUES LOEB.

(From the Rudolf Spreckels Physiological Laboratory of the University of California.)

(Received for publication, January 26, 1906.)

In a former paper on the rhythmical contractions of striped muscle¹ I have shown that magnesium as well as calcium inhibits the rhythmical contractions which appear in a pure sodium chloride or lithium chloride solution and that the same seems true for the rhythmical contractions of a jellyfish of the Atlantic (*Gonionemus*).² In this paper I intend to show that the normal rhythmical contractions of *Polyorchis*, a jellyfish of the Bay of San Francisco, are caused by magnesium or are at least only possible in solutions containing magnesium; and that the contractions of the isolated centre of this medusa (*i. e.*, the medusa deprived of its margin containing the sense organs and the central nervous system) are caused by calcium. In addition it will be shown that calcium and magnesium are antagonistic to each other.

I. PROOF THAT THE "SPONTANEOUS" SWIMMING MOTIONS OF POLYORCHIS DO NOT OCCUR EXCEPT IN THE PRESENCE OF MAGNESIUM.

Sea-water is, according to van't Hoff, a mixture of 100 mol. $MgCl_2$, 2.2 mol. KCl , about 2 mol. $CaCl_2$, 7.8 mol. $MgCl_2$, and 3.8 mol. $MgSO_4$. For animals living in the bay $\frac{3}{8}$ gram-molecular solutions are appropriate and such solutions are used in the following experiments unless the contrary is stated.

If we make all possible combinations of one, two, three, or

¹ *Festschrift für Professor Fick*, Würzburg, 1899 (Reprinted in *Studies in General Physiology*, ii, p. 518, 1905, Chicago).

² *Amer. Journ. Physiol.*, iii, p. 383, 1900 (Reprinted in *Studies in General Physiology*, ii, p. 559).

four of these constituents we notice that the "spontaneous," characteristic swimming motions occur only in those solutions which contain magnesium. In order to simplify matters we shall omit the SO_4 from consideration since I have convinced myself that the presence or absence of SO_4 does not alter the result. Among the following list of solutions, namely,

1. 50 c.c. NaCl
2. " " " + 1 c.c. KCl
3. " " " + 1 c.c. CaCl_2
4. " " " + " " + 1 c.c. KCl
5. " " " + 6 c.c. MgCl_2
6. " " " + " " + 1 c.c. KCl
7. " " " + " " + 1 c.c. CaCl_2
8. " " " + " " + " " + 1 c.c. KCl

only solutions 5, 6, 7, and 8 will allow the normal, "spontaneous," swimming motions of *Polyorchis*. In solutions 1, 2, 3, and 4 no motions will occur, as a rule, and the mouth of the animal will contract. If occasional motions occur they are confined to the muscles of the umbrella and are the somewhat uncoordinated motions of the isolated centre, which we shall discuss in a later section. In these experiments care must be taken to remove all traces of sea-water from the inside and outside surface of the animal with the aid of filter paper and by washing in a pure sodium chloride solution before the experiment is tried.

In 50 c.c. NaCl + 6 c.c. MgCl_2 , the animals show very regular, rhythmical, swimming motions for from one to three hours. The animal may remain alive for several days in this solution although all contractions have ceased. When put back into normal sea-water, the contractions will begin again in a few minutes.

In a mixture of 50 c. c. NaCl + 6 c. c. MgCl_2 + 1 c. c. CaCl_2 the rhythmical spontaneous contractions will continue for a day or two days. If we increase the quantity of calcium chloride in the above-mentioned solution or in normal sea-water, the swimming motions will be partly, and finally completely inhibited. To 50 c. c. of sea-water were added 0, 1, 2, 3, 4, 5, 10, 20, and 30 c. c. of $\frac{1}{8}\text{M}$ CaCl_2 solution. Upon the addition of 1 to 4 c. c. of calcium chloride the swimming motions occur with a latent period, increasing with the amount of calcium; the addition of 5 c. c. as a rule suffices to completely suppress the

swimming motions, except for a short period of rhythmical activity at the beginning.

The amount of calcium chloride required to suppress the swimming motions depends upon the amount of magnesium in solution. In a mixture of 50 c. c. of NaCl + 5 c. c. of MgCl_2 the contractions were suppressed through the addition of about 5 c. c. of CaCl_2 , while in a mixture of 50 c. c. of NaCl + 10 c. c. of MgCl_2 it required about 10 c. c. of CaCl_2 to inhibit the rhythmical contractions.

In a pure sodium chloride solution no spontaneous swimming motions will occur (provided all the sea-water is washed off from the inside of the swimming bell) and the results remain the same or become still more pronounced, if we add increasing amounts of calcium chloride. If we add, however, increasing amounts of magnesium chloride we find that with the increase of magnesium the swimming motions become more rapid. In normal sea-water the rhythmical contractions appear in groups with pauses between them. When we increase the amount of magnesium chloride these pauses disappear. But there is a limit to which the concentration of the magnesium chloride may be raised. If we add 10 c. c. of $\frac{3}{8}\text{M}$ MgCl_2 to 50 c. c. of sea-water the spontaneous motions may occur for about two days, going on almost incessantly. If we add 20 c. c. of MgCl_2 the swimming motions are very rapid but cease soon on account of a secondary paralyzing effect of the magnesium chloride upon the muscles which will be discussed later.

When we add to a mixture of 50 c. c. of $\frac{3}{8}\text{M}$ NaCl + 6 c. c. of $\frac{3}{8}\text{M}$ MgCl_2 , 0, 1, 2, 4, 8, 16, c. c. of $\frac{3}{8}\text{M}$ KCl, swimming motions (or normal rhythmical contractions) will only occur in the solutions containing less than 4 c. c. of KCl. In 50 c. c. of NaCl + 6 c. c. of MgCl_2 + 1 c. c. of KCl the regular motions will begin almost instantly and will continue longer than without potassium chloride, but not as long as with calcium chloride. When we add the same quantities of potassium chloride to 50 c. c. of NaCl alone, without magnesium, no swimming motions will occur; if motions occur at all, they are the uncoordinated muscular or fibrillary contractions of the centre with which we shall deal later.

If we add both 1 c. c. of $\frac{3}{8}\text{M}$ KCl and 1 c. c. of $\frac{3}{8}\text{M}$ CaCl_2 to

50 c. c. of $\frac{3}{8}M$ NaCl + 6 c. c. of $\frac{3}{8}M$ MgCl₂ the spontaneous contractions will continue indefinitely, *i. e.*, as long as in sea-water. It seems that the addition of potassium accelerates the quickness of the contractions slightly; at least after twenty-four hours such a difference is noticeable.

In all these experiments the magnesium acted in the presence of sodium chloride. The question arose whether or not it is possible to substitute other substances for sodium chloride. Lithium chloride can take the place of sodium chloride except that lithium chloride is rather toxic and it is not possible to keep the animals alive more than twenty-four hours (probably not even as long). Everything occurs in lithium chloride as in the case of sodium chloride. No swimming motions were, however, observed when magnesium was added to a solution of sodium acetate or butyrate. Sodium sulphate was only slightly better; sodium citrate, bicarbonate, and ammonium nitrate gave practically negative results.

It was, however, possible to produce normal and regular rhythmical contractions which lasted five minutes or more when 15 c. c. of $\frac{3}{8}M$ MgCl₂ were added to 50 c. c. of $\frac{3}{8}M$ cane sugar solution. No other substance but magnesium acted in this way. The cane sugar used in these experiments (and those to be mentioned later) was the purest obtainable, was recrystallized in the laboratory and tested with negative results for sodium, calcium, barium, and strontium.

Experiments with urea as a substitute for sodium chloride gave practically negative results. In pure magnesium chloride solutions a few contractions were produced when 15 c. c. of $\frac{3}{8}M$ MgCl₂ were added to 50 c. c. of water. The fact that the contractions did not last longer may be due to the degree of dilution, as experiments with diluted sea-water indicated. If the concentration of magnesium chloride is greater, the paralyzing effects of magnesium upon the muscle occur too rapidly.

These and a number of other experiments not mentioned here show that the spontaneous normal, swimming motions of *Polyorchis* occur only in the presence of magnesium and that their occurrence in sea-water is due to the presence of magnesium in a rather high concentration. There are at least two possible causes for this remarkable action of magnesium chloride. The

latter may either stimulate the sense organs or the nerves of the margin and thus be the direct cause of the "spontaneous" contractions, or it may act indirectly by keeping the muscles of the edge in a relaxed condition and thus guarantee the relaxation after a systole. In the latter case we should have to assume that some other ion (*e. g.*, sodium, potassium, calcium, etc.) causes the muscles to contract, but that, in the absence of magnesium, this will result in a tonic, steady contraction of the margin, while the magnesium causes a relaxation of the muscles of the margin which favors the diastole. In favor of the latter view may be mentioned the fact that the mouth and tentacles are permanently contracted in any solution without magnesium and that the tentacles are, as a rule, the more relaxed, the more magnesium (within certain limits) the solution contains. The experiments thus far made allow no decision between the two possibilities mentioned.

II. THE STIMULATING EFFECT OF CALCIUM, STRONTIUM, AND BARIUM UPON THE ISOLATED CENTRE OF POLYORCHIS, AND THE INHIBITORY EFFECT OF MAGNESIUM.

Romanes found that if the margin containing the sense organs and the central nervous system of a medusa be cut off, the centre is no longer able to beat in sea-water. In *Gonionemus* I found that this is due to the presence of calcium and magnesium in the sea-water, since the centre of *Gonionemus* will beat in a pure solution of sodium chloride but will not beat when calcium chloride and magnesium chloride are added in the proper quantity. Barium is still more effective than sodium chloride in producing rhythmical contractions. I expected that calcium would act in the same way, but was not able to test this on *Gonionemus* which does not occur in the bay. In *Polyorchis* it can be easily shown that a pure solution of calcium chloride will cause the isolated centre to beat rhythmically. The centre of *Polyorchis* will not beat in normal sea-water and acts in this respect like the centre of *Gonionemus*, but it differs from the latter in that it will not beat for some time when put into a pure sodium chloride solution. As a rule more than three hours—often a whole day—may elapse before the centre of *Polyorchis* begins to beat in sea-water. After this the beats may continue, with

long intermissions, for a whole day. When we isolate the centre of *Polyorchis* and put it into 50 c. c. of a pure $\frac{3}{8}^M$ cane sugar solution no contractions will occur, no matter how long we wait. But if we add from 10 to 15 c. c. of $\frac{3}{8}^M$ CaCl_2 solution the contractions will begin almost instantly, and will continue for several hours. If we add less calcium chloride, contractions will also begin, but with an initial delay which is the greater the smaller the amount of calcium added. The calcium used in this case was freed from sodium and the centres were washed most carefully, sometimes for an hour, in a pure cane sugar solution before the calcium was added, to make sure that all the sodium was removed from the surface of the animal. I convinced myself also that the addition of small or large quantities of sodium chloride did not increase the stimulating effect of calcium.

The calcium acts in the same way when added to a sodium chloride solution. When a centre is put into 50 c. c. of $\frac{3}{8}^M$ NaCl no contractions will, as a rule, occur, but if from 10 to 15 c. c. of $\frac{3}{8}^M$ CaCl_2 are added contractions will promptly occur. The centre which will not beat in sea-water will promptly beat when we add from 20 to 30 c. c. of $\frac{3}{8}^M$ CaCl_2 to 50 c. c. of sea-water, and these beats will last for a number of hours.

It requires much less barium chloride than calcium chloride to call forth immediately rhythmical contractions of the isolated centre in a sugar solution. When 1 c. c. of $\frac{1}{8}^M$ BaCl_2 is added to 50 c. c. of $\frac{3}{8}^M$ cane sugar, the contractions will begin immediately and will last as long as ten minutes. The addition of small amounts of sodium chloride to the solution does not modify the result. If 0.5 c. c. of $\frac{1}{8}^M$ BaCl_2 is added the contractions will not begin until after two or three minutes, but may last almost thirty minutes. The experiments show the great stimulating power as well as toxicity of barium.

Strontium chloride also calls forth the rhythmical contractions of the centre when added to sea-water, sodium chloride solution, sugar solution, or distilled water. When 8 c. c. of $\frac{3}{8}^M$ SrCl_2 are added to 50 c. c. of $\frac{3}{8}^M$ cane sugar, contractions of the centre begin immediately. They do not last as long as those produced by calcium.

It was practically, if not absolutely, impossible to produce

rhythmical contractions of the centre by magnesium chloride. Whether the magnesium chloride was added to sea-water, sodium chloride, or sugar solutions or to distilled water, the result was equally negative. Occasionally a couple of contractions were observed in such solutions. It could be shown, however, that magnesium chloride directly inhibits the stimulating effect of calcium. If to a mixture of 50 c. c. of NaCl + 15 c. c. of CaCl_2 0, 15, 30, 45, and 60 c. c. of MgCl_2 are added, it will be noticed that in the solution with 60 c. c. of MgCl_2 no contractions of the centre occur, while in the solutions with 30 and 45 c. c. of MgCl_2 they will begin after some delay, and in the first two solutions they will begin almost instantly. Magnesium chloride will therefore inhibit the stimulating action of calcium chloride upon the centre when the two salts are in the ratio of about 4:1. In the sea-water they are contained in the ratio of about 5:1 and this is partly the reason that the centre does not beat in it spontaneously.

We have stated in the previous section that calcium chloride inhibits the normal swimming movements of *Polyorchis*. If we add about 20 c. c. of $\frac{3}{8}M$ CaCl_2 to 50 c. c. of sea-water, after some time (about an hour or a little sooner) contractions will occur in the normal jellyfish, but these contractions are those occurring also in the isolated centre and are not the normal swimming movements of the uninjured animal. The same type of contractions may be observed also in any solution free from magnesium or where the calcium is in excess of the magnesium. When we make an incision into the swimming bell of *Polyorchis*, calcium produces these contractions much more quickly than without such an incision, presumably because in the former case it enters more quickly into the circulation of the animal.

III. THE STIMULATING EFFECT OF THE DECALCIFYING SALTS AND OF ACID UPON THE CENTRE OF POLYORCHIS.

In a former paper I have shown that the decalcifying salts like oxalates, fluorides, citrates, etc., are stimulants for the centre of *Gonionemus* and other, possibly all, contractile organs. It can be shown that the centre of *Polyorchis* is no exception to this rule since the decalcifying salts cause it to make rhythmical

contractions. If about 10 c. c. of a gram-molecular solution of sodium citrate are added to 50 c. c. of $\frac{3}{8}^M$ NaCl, rhythmical beats will be produced almost instantly which may last an hour or longer. If the citrate is added to a sugar solution a little more has to be used. Sodium oxalate is much more effective than the citrate. If 8 c. c. of an approximately $\frac{N}{2}$ sodium oxalate solution are added to 50 c. c. of a cane sugar solution, contractions of the centre will begin at once and last for a few minutes. Sodium oleate is more effective but also more toxic than either of the preceding salts. Sodium bicarbonate did not act, on account of its alkalinity, as we shall see later; the action of sodium sulphate was also slight. Sodium fluoride was very effective but also very toxic.

We are therefore dealing with the paradoxical result that decalcifying salts as well as calcium cause the isolated centre to beat. We are evidently dealing in all cases of stimulation by salts with very complicated conditions of chemical equilibrium between the salts added and the salts and ion-proteid compounds or other ion-colloid compounds in the cells. It can also be shown that acids cause the isolated centre of *Polyorchis* to beat, while alkalis have the opposite effect. When 0.3 c. c. of $\frac{N}{10}$ HCl is added to 50 c. c. of $\frac{3}{8}^M$ NaCl, the centre begins to beat after one minute and may continue to beat twenty minutes. When 0.2 c. c. was added the centre began to beat after fifteen minutes and the beats lasted thirty-five minutes. When 0.05 c. c. of HCl was added, the beats began at once but lasted only one or two minutes. With more hydrochloric acid only a few contractions occurred. If carbon dioxide is added to the sodium chloride solution containing isolated centres, it also causes them to beat. The addition of sodium hydroxide had no such effect, no matter how much or how little was added. Acid has the same stimulating effect when added to sea-water, only much more acid must be used than in sodium chloride partly on account of the neutralizing effect of the carbonates and phosphates in the sea-water, and partly perhaps, in order to overcome the inhibiting effect of the magnesium chloride contained in the sea-water. The best results were obtained when from 0.75 to 0.9 c. c. of $\frac{N}{10}$ HCl was added to 50 c. c. of sea-water. In such solutions the

centre may beat for an hour. I was surprised to notice that in such sea-water the isolated centres may keep alive for several days. When sodium hydroxide was added to sea-water no contractions could be produced in the isolated centre of *Polyorchis*. It was also very striking that in alkaline sea-water the centres died much more rapidly than in acid or neutral sea-water.

Readers who are familiar with the papers of Loevenhart¹ and Van Slyke and Hart² on the coagulation of milk and the nature of the changes occurring in casein or paracasein under such conditions cannot fail to notice several points of coincidence between the actions of electrolytes in that case and the stimulating and inhibitory effects of salts in the experiments mentioned in this paper. I do not consider it profitable, however, to dwell further on this matter for the present than to indicate the complicated character of the changes that must occur in a cell when a salt enters.

In conclusion I wish to point out that an addition of magnesium to sea-water is considerably less injurious than the addition of an equivalent amount of calcium. The difference is as striking as that between the addition of acid and alkali.

IV. SUMMARY OF RESULTS.

1. The experiments show that the normal rhythmical swimming motions of a jellyfish (*Polyorchis*) will only occur in such solutions as contain magnesium and that the apparently spontaneous character of these rhythmical motions depends upon the magnesium contained in the sea-water.

2. This effect of magnesium can be inhibited by the addition of an equivalent amount of calcium or potassium.

3. The isolated centre of *Polyorchis*, which will not beat in a pure sugar solution or in sea-water, can be caused to beat in both solutions through the addition of a certain amount of calcium chloride, (or strontium chloride or barium chloride) but not by magnesium chloride. Magnesium inhibits the stimulating effect of calcium when magnesium is added in the ratio of at least four times the equivalent of calcium.

¹ *Zeitschr. f. physiol. Chem.*, xli, p. 177, 1904.

² *Amer. Chem. Journ.*, xxxiii, p. 461, 1905.

4. The isolated centre of *Polyorchis* will as a rule not beat or only after a number of hours in a pure sodium chloride solution, but it will beat instantly for from one to three hours when put into a solution of calcium chloride in cane sugar.

5. The isolated centre of *Polyorchis* can be caused to beat by any of the decalcifying salts, *e. g.*, oxalates, fluorides, oleates, citrates, etc.

6. Acids will cause the isolated centre of *Polyorchis* to beat while alkalies inhibit such beats.

II. RESEARCHES ON PYRIMIDINS: ON METHODS OF SYNTHESIZING ISOBARBITURIC ACID, AND 5-OXY-CYTOSIN.

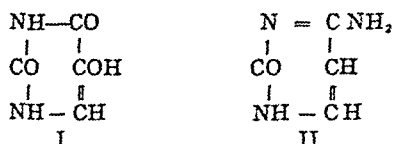
(Fourteenth Paper.)

BY TREAT B. JOHNSON AND ELMER V. MCCOLLUM.

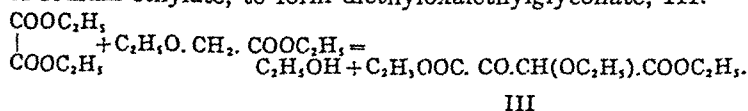
(From the Sheffield Laboratory of Yale University.)

(Received for publication, January 2, 1906.)

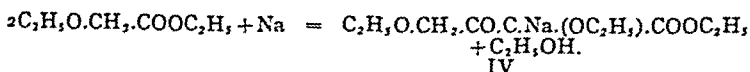
In this paper we describe a new synthesis of isobarbituric acid, I. In the course of the work we have also prepared several new derivatives of this acid and of cytosin, II, which are of especial interest.



Wislicenus and Scheidt¹ have shown that diethyloxalate condenses with the ethyl ester of ethylglycollic acid, in presence of sodium ethylate, to form diethyloxalethylglycollate, III.



It was also observed by Conrad² that diethylglycollate undergoes an inner condensation, in benzene, in the presence of metallic sodium to form the sodium salt of ethyl α, γ -diethoxyacetoacetate³, IV.



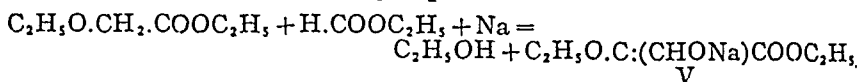
It might be expected that ethylformate would also condense

¹ *Ber. d. deutsch. chem. Gesellsch.*, xxiv, p. 432, 1891.

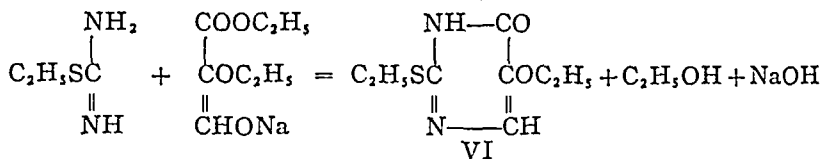
² *Ibid.*, xi, p. 58, 1878.

³ *Ann. d. Chem. (Liebig)*, cclxix, p. 28, 1892.

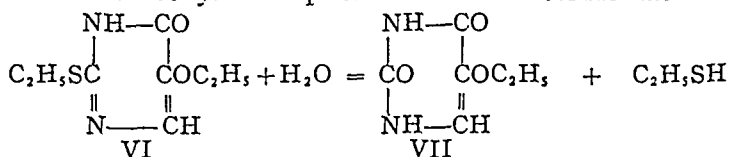
with diethylglycollate in the presence of metallic sodium to form the sodium salt of ethyl- α -ethoxy- β -oxyacrylate,¹ V. We find that this condensation takes place in the normal manner according to the following equation:



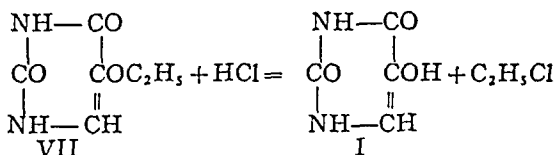
When this sodium salt, V., was dissolved in water with the calculated quantity of pseudoethylthiourea they reacted to form 2-ethylmercapto-5-ethoxy-6-oxypyrimidin, VI., as follows:



This mercaptopyrimidin, VI., was converted practically quantitatively into isobarbituric acid, I., when heated with hydrochloric acid. This change involves two distinct reactions, as follows: The 2-ethylmercapto-5-ethoxy-6-oxypyrimidin, VI., is first converted into 2,6-dioxy-5-ethoxypyrimidin, VII., with evolution of ethylmercaptan. This intermediate ethoxy-



pyrimidin, VII., then reacts with hydrochloric acid to give isobarbituric acid, I., with formation of ethylchloride.

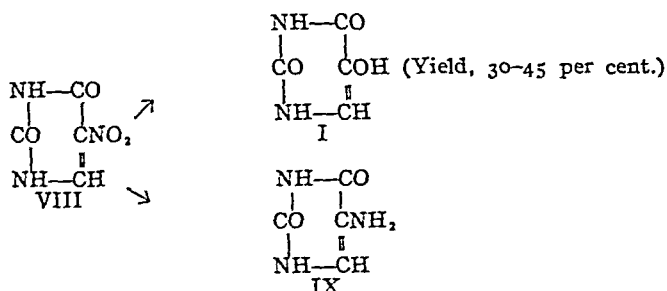


The only method, given in the literature, for preparing isobarbituric acid is that described by Behrend.² He prepared it by reducing nitrouracil, VIII., with tin and hydrochloric acid.

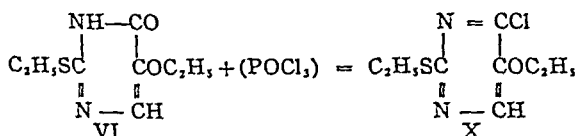
¹We find that ethylformate condenses with ethylphenoxycetate— $\text{C}_6\text{H}_5\text{O} \cdot \text{CH}_2 \cdot \text{COOC}_2\text{H}_5$ —to give a quantitative yield of the sodium salt of ethyl- α -phenoxy- β -oxyacrylate— $\text{C}_6\text{H}_5\text{O} \cdot \text{C} : (\text{CHONa}) \cdot \text{COOC}_2\text{H}_5$. We shall condense ethylformate with other glycollic esters and study some of the reactions of this new class of substituted acrylic esters. (T. B. J.)

²*Ann. d. Chem.* (Liebig), cxxix, p. 39; Behrend and Roosen, *ibid.*, ccli, p. 239.

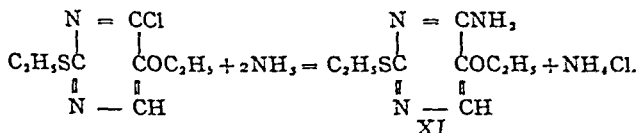
This method of preparation is not suitable for preparing large quantities of the acid. Part of the nitrouracil is recovered in the form of a minouracil, IX., and the yield of isobarbituric acid, I., corresponds to only about thirty to forty-five per cent. of the theoretical.



When 2-ethylmercapto-5-ethoxy-6-oxypyrimidin, VI., was warmed with phosphorus oxychloride, it was converted smoothly into 2-ethylmercapto-5-ethoxy-6-chlorpyrimidin, X.



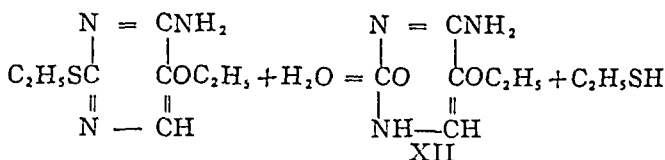
This chlorpyrimidin, X., gave a theoretical yield of 2-ethylmercapto-5-ethoxy-6-aminopyrimidin, XI., when heated with strong alcoholic ammonia:



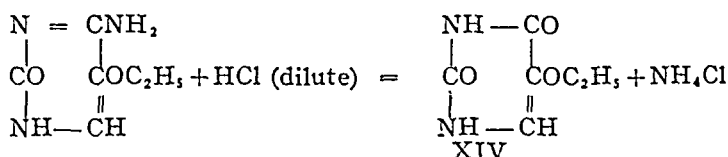
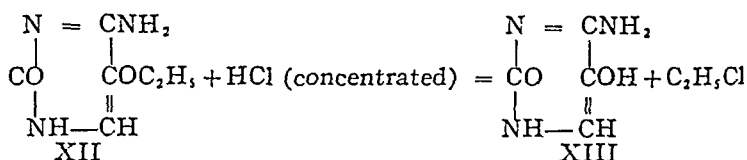
Especially interesting was the behavior of this aminopyrimidin, XI., towards hydrochloric acid. When heated with this reagent, it was converted into isobarbituric acid. We have investigated the mechanism of this reaction and find that it involves the formation of several intermediate compounds. We have succeeded in isolating each intermediate product of the reaction.

2-Ethylmercapto-5-ethoxy-6-aminopyrimidin, XI., is first

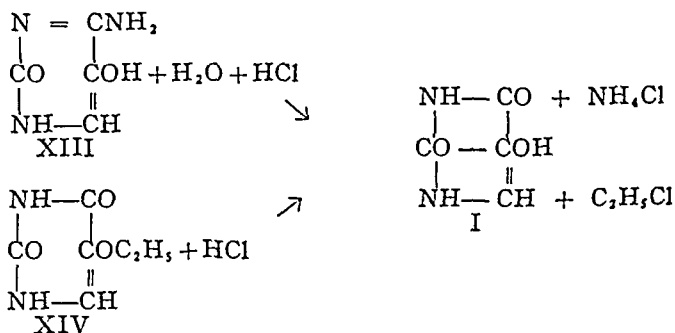
converted into 2-oxy-5-ethoxy-6-aminopyrimidin or 5-ethoxycytosin, XII.



5-Ethoxycytosin, XII., then reacts in two ways, according to the strength of the hydrochloric acid used, giving 2,5-dioxy-6-aminopyrimidin or 5-oxycytosin, XIII., and 2,6-dioxy-5-ethoxypyrimidin, XIV., as follows:



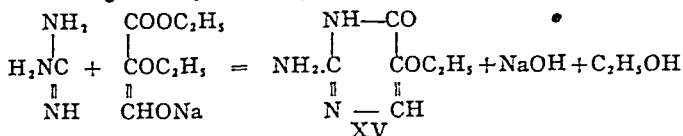
5-Oxycytosin, XIII., and 2, 6-dioxy-5-ethoxypyrimidin, XIV., then react with an excess of hydrochloric acid to form isobarbituric acid according to the following equations:



The sodium salt of ethyl- α -ethoxy- β -oxyacrylate, V., can also be used for preparing 5-oxy-derivatives of isocytosin.¹ Dr. C. O. Johns finds that this salt condenses with guanidin to

¹Wheeler and Johnson, *Amer. Chem. Jour.*, xxix, p. 492, 1903.

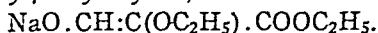
give 2-amino-5-ethoxy-6-oxypyrimidin or 5-ethoxyisocytosin, XV.



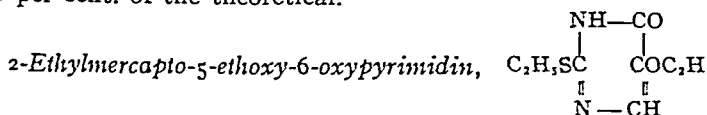
We shall continue the study of these interesting pyrimidin derivatives in this laboratory.

EXPERIMENTAL PART.

Sodium Salt of Ethyl- α -ethoxy- β -oxyacrylate,



This salt was prepared by slowly adding to ether, in which was suspended the calculated quantity of sodium, a mixture of molecular proportions of ethylformate and the ethyl ester of ethylglycolic acid. The condensation proceeded smoothly with evolution of hydrogen gas. In the course of 8 to 12 hours, the sodium all dissolved. We made no attempts to isolate the sodium salt. It was hygroscopic and very soluble in ether. After the condensation was complete, the excess of ether was evaporated in a partial vacuum and the crude salt dissolved in water. This aqueous solution was used for experiments that we describe in this paper. We have assumed, for the purpose of calculation, that the yield of sodium salt corresponds to about 90 per cent. of the theoretical.



This pyrimidin was prepared as follows: 80 grams of the hydrobromic acid salt of pseudoethylthiourea were dissolved in about 150 c.c. of cold water and added to a strong aqueous solution containing two molecular proportions of the sodium salt of ethyl- α -ethoxy- β -oxyacrylate. This solution was then combined with 50 c.c. of water containing a molecular proportion of potassium hydroxide (24.5 grams). The mixture was then thoroughly shaken; allowed to stand one hour at ordinary temperature, and then heated on the steam-bath for another hour. It was then thoroughly cooled and acidified with acetic acid. An excess of acetic acid should be avoided as the pyrim-

idin is somewhat soluble in this reagent. The mercaptopyrimidin separated at once as a brown, crystalline product. The crude material melted at 160° – 165° C. The yield was 50 grams, or 61 per cent. of the theoretical, calculating from the weight of pseudothiourea used. This yield of pyrimidin was not increased when we used more than a one-half molecular proportion of the hydrobromic acid salt of the pseudothiourea for the condensation. Long standing of the alkaline solution also does not increase the yield of pyrimidin. In one experiment the solution was divided into three equal parts and treated as follows: one part was warmed immediately on the steam-bath for one hour and then acidified with acetic acid; the second part was allowed to stand two hours, and the third twenty-four hours before warming on the steam-bath and acidifying with acetic acid. The yields of pyrimidin from the three equal portions were practically the same. The pyrimidin is difficultly soluble in hot water and practically insoluble in the cold. It deposited from hot alcohol in rhombic-shaped prisms and melted at 169° C. to a clear oil. Analysis (Kjeldahl):

0.4170 gram of substance gave 0.05712 gram of nitrogen = 40.8 c.c. $\frac{1}{16}$ normal HCl.

Calculated for $C_8H_{12}O_2N_2S$:
N = 14.00 per cent.

Found.
13.70 per cent.

Action of Hydrochloric Acid on 2-Ethylmercapto-5-ethoxy-6-oxy-pyrimidin.—The mercapto-radical in this pyrimidin is very firmly bound and cannot be removed in the usual manner by boiling with hydrochloric acid. The mercaptopyrimidin was recovered unaltered after boiling for several hours with this acid. It was also recovered unaltered (melting at 168° C.) after boiling with redistilled hydrobromic acid for six hours.

Action of Twenty Per Cent. Hydrochloric Acid at 150° C.—Ten grams of the mercaptopyrimidin were heated with an excess of twenty per cent. hydrochloric acid for four hours at 150 – 155° C. When the tube was opened there was considerable pressure due to the presence of ethylchloride and ethylmercaptan floated on the surface of the liquid. A colorless, crystalline product was suspended in the solution. It was insoluble in cold water. It deposited from hot water in the form of balls of microscopic prisms. It had no definite decomposition point and did not contain sulphur. A nitrogen determination indicated a mixture

of isobarbituric acid and 2,6-dioxy-5-ethoxypyrimidin. Analysis (Kjeldahl):

0.1363 gram of substance gave 0.02646 gram of nitrogen = 18.9 c.c. $\frac{1}{10}$ normal HCl.

Calculated for $C_4H_4O_3N_2$.	Calculated for $C_6H_6O_4N_2$.	Found.
N = 21.87 per cent.	17.92 per cent.	19.3 per cent.

We did not have enough material to separate the ethoxy-derivative sufficiently pure for analysis. In order to separate the isobarbituric acid from any ethoxy-derivative the mixture was dissolved in boiling acetic anhydride. Upon cooling the acetyl-derivative¹ of isobarbituric acid was obtained in the form of well-developed prisms. It crystallized from hot water in the form of radiating prisms. Analysis (Kjeldahl):

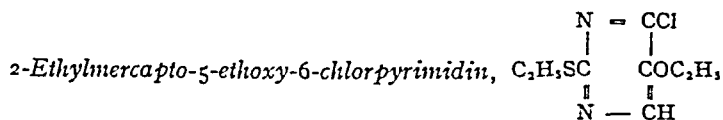
0.1162 gram of substance gave 0.01897 gram of nitrogen = 13.55 c.c. $\frac{1}{10}$ normal HCl.

Calculated for $C_6H_6O_4N_2$.	Found.
N = 16.47 per cent.	16.33 per cent.

Action of Concentrated Hydrochloric Acid at 150° C.—When the mercaptopyrimidin was heated with concentrated hydrochloric acid, it was converted smoothly into isobarbituric acid. Four grams of the mercaptopyrimidin were heated with concentrated hydrochloric acid for three hours at 145° to 155° C. When the tube was opened there was great pressure (ethylchloride) and ethylmercaptan was recognized by its stench. The solution was evaporated to dryness to remove the excess of hydrochloric acid. The crystalline residue was then purified for analysis by repeated recrystallizations from hot water. It separated in corpuscular crystals and decomposed above 300° C., without melting. A nitrogen determination agreed with the calculated in isobarbituric acid.

0.0590 gram of substance gave 0.01288 gram of nitrogen = 9.2 c.c. $\frac{1}{10}$ normal HCl.

Calculated for $C_4H_4O_3N_2$.	Found.
N = 21.87 per cent.	21.83 per cent.



Thirty grams of 2-ethylmercapto-5-ethoxy-6-oxypyrimidin

¹ Behrend and Roosen, *loc. cit.*

were warmed with 50 c. c. of phosphorus oxychloride. Hydrochloric acid began to be evolved immediately, and after heating a few minutes on the steam-bath the reaction was complete. The excess of phosphorus oxychloride was then removed by heating at 100° C. under diminished pressure. We obtained a double compound of phosphorus oxychloride and the chlorpyrimidin that was not decomposed by cold water. It was insoluble in water and did not solidify on standing. In order to isolate the chlorpyrimidin it was necessary to decompose the phosphorus compound with hot water. By this treatment the chlorpyrimidin was obtained as an oil which finally solidified. It was dissolved in ether; the ethereal solution dried over calcium chloride and purified by distillation under diminished pressure. Almost the entire product boiled constant at 185° C. under a pressure of 25 millimeters. On cooling, it crystallized in beautiful, radiating prisms which melted at 44°–46° C. It crystallized from ligroin in well developed prisms that melted at 46° C. The chloride could be boiled with water for hours without any apparent change. It was soluble in cold alcohol and benzene. The yield of distilled product was 24 grams or 73 per cent. of the theoretical. Analysis (Kjeldahl):

0.2446 gram of substance gave 0.0308 gram of nitrogen = 22. c.c. $\frac{1}{16}$ normal HCl.

0.1605 gram substance gave 18.9 c.c. nitrogen gas at 24° and 757 mm.

Calculated for $C_8H_{11}ON_2SCl$.	Found.	
	I.	II.
N = 12.81 per cent.	12.59	13.1 per cent.
$ \begin{array}{c} \text{N} = \text{CNH}_2 \\ \qquad \qquad \\ \text{2-Ethylmercapto-5-ethoxy-6-aminopyrimidin, } C_8H_{11}SC \qquad COC_2H_5 \\ \qquad \qquad \\ \text{N} - \text{CH} \end{array} $		

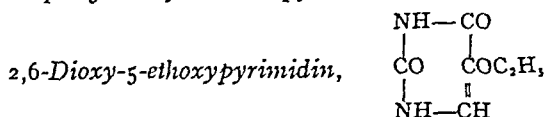
Fourteen grams of 2-ethylmercapto-5-ethoxy-6-chlorpyrimidin were heated with an excess of concentrated alcoholic ammonia for three hours at 150°–160° C. Under these conditions the aminopyrimidin was obtained as a well crystallized solid. It was insoluble in cold water and alcohol. It deposited from hot water or alcohol in rhombic-shaped prisms that melted at 105° C. to a clear oil. The yield was quantitative. When we attempted to prepare this base by heating with alcoholic ammonia

at 120°–130° C. about one-half of the chlorpyrimidin was recovered unaltered. Analysis:

Calculated for $C_8H_{11}ON_2S$.
N = 21.10 per cent.

Found.
21.00 per cent.

Action of Twenty Per Cent. Hydrochloric Acid on 2-Ethylmercapto-5-ethoxy-6-aminopyrimidin:

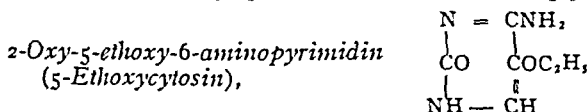


Three grams of the aminopyrimidin were heated in a sealed tube with an excess of 20 per cent. hydrochloric acid for four hours at 150°–160° C. When the tube was examined there was little pressure and drops of mercaptan were suspended in the liquid. The solution was filtered and allowed to evaporate spontaneously in a vacuum. After about three-fourths of the liquid had evaporated, a small amount of crystalline material separated. This was filtered off and crystallized from hot water. It deposited in balls of microscopic prisms and agreed in its behavior with that of isobarbituric acid. We did not obtain sufficient material for analysis. The acid filtrate was evaporated on the steam-bath to about 10 c.c. On cooling, a well-crystallized product deposited. This was filtered off and the filtrate saved (see 5-ethoxycytosin.) It crystallized from hot water in aggregates of radiating prisms. It began to turn brown at about 220° C. and then decomposed from 260° to 280° C. with effervescence according to the rate of heating. It was insoluble in acids and did not respond to a test for sulphur. Analysis (Kjeldahl):

0.0881 gram of substance gave 0.01617 gram of nitrogen = 11.5 c. c. $\frac{1}{10}$ normal HCl.

Calculated for $C_8H_9O_2N_2$.
N = 17.94 per cent

Found.
18.3 per cent.



The acid filtrate, after filtering from the 2, 6-dioxy-5-ethoxypyrimidin described above, was allowed to evaporate to dryness at ordinary temperature. There remained a crystalline residue

which dissolved in cold water to a clear solution. When this was made distinctly alkaline with ammonium hydroxide, we obtained a beautiful deposit of slender prisms. They melted sharply at 300° C. to a black oil. The compound was very soluble in warm water. It was free from sulphur and did not contain water of crystallization. Analysis:

Calculated for $C_6H_7O_2N_3$.
N = 27.09 per cent.

Found.
27.22 per cent.

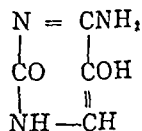
Picrate of 5-Ethoxycytosin.—This was obtained when picric acid was added to a solution of the base. It deposited in characteristic, arborescent crystals. When slowly heated, it melted at 229° – 231° C. to a clear oil. Analysis:

Calculated for $C_6H_7O_2N_3 \cdot C_6H_3O_7N_3$.
N = 21.87 per cent.

Found.
22.00 per cent.

Action of Concentrated Hydrochloric Acid on 2-Ethylmercapto-5-ethoxy-6-aminopyrimidin.

2, 5-Dioxy-6-aminopyrimidin (5-Oxycytosin)



Three grams of 2-ethylmercapto-5-ethoxy-6-aminopyrimidin were heated in a sealed tube with concentrated hydrochloric acid at 150° C. for two hours. When the tube was opened there was great pressure (ethylchloride) and a strong odor of ethylmercaptan. The contents of the tube were evaporated on the steam-bath to a syrup and then dissolved in 10 c.c. of cold water. When this aqueous solution was made alkaline with ammonia we obtained a crystalline precipitate. It was purified by first boiling with alcohol to remove any unaltered mercaptopyrimidin and then crystallizing from water. It deposited in prismatic crystals that did not melt below 280° C. On account of the small amount obtained of the base, we were unable to free it from impurities. Nevertheless a nitrogen determination indicated that we were dealing with a 2, 5-dioxy-6-aminopyrimidin:

Calculated for $C_4H_4O_2N_2$.
N = 33.0 per cent.

Found.
32.20 per cent.

This experiment was repeated under the following conditions: Five grams of the mercaptopyrimidin were heated with concentrated hydrochloric acid for three hours at 150° – 160° C. Ethylchloride and ethylmercaptan were formed as in the previous

experiment. The contents of the tube were evaporated to dryness on the steam-bath. We obtained a crystalline residue that partly dissolved in cold water. The insoluble material was filtered off and crystallized from hot water. It was difficultly soluble and deposited on cooling in balls of microscopic prisms. It had no definite melting point and did not contain sulphur. A nitrogen determination agreed with the calculated value in isobarbituric acid.

Calculated for $C_4H_4O_3N_2$.

N = 21.87 per cent.

Found.

22.3 per cent.

The aqueous filtrate above, after filtering from isobarbituric acid, was made alkaline with ammonium hydroxide. We obtained no precipitate at first, but after a few hours microscopic prisms deposited which were identified as isobarbituric acid. The alkaline solution was again filtered and then combined with a strong solution of picric acid. We obtained a well-crystallized picrate. It deposited from hot water in well-developed prisms arranged in crosses. When heated in a capillary tube it sintered above 220° C., then slowly decomposed above 240° C. but did not effervesce at 270° C. It was free from sulphur and did not contain water of crystallization. A nitrogen determination agreed with the calculated value in the picrate of 2,5-dioxy-cytosin (Kjeldahl):

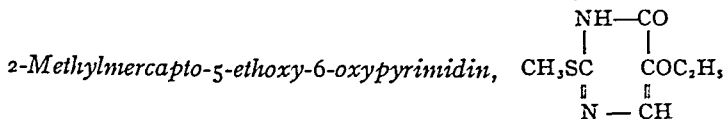
0.1067 gram of substance gave 0.02506 gram of nitrogen = 17.9 c.c. $\frac{1}{16}$ normal HCl.

Calculated for $C_4H_4O_3N_2 \cdot C_6H_3O_3N_3$.

N = 23.59 per cent.

Found.

23.48 per cent.



Seventy-five grams of the hydriodic acid salt of pseudomethylthiourea were dissolved in water and added to a solution of 120 grams of the sodium salt of ethyl- α -ethoxy- β -oxyacrylate. To this solution was then added a molecular proportion of potassium hydroxide (19.5 grams) which was first dissolved in water. The mixture was then allowed to stand for four hours; warmed on the steam-bath for one hour, and then acidified with acetic acid. The solution was then concentrated to about one-half its original volume and thoroughly cooled. The

mercapto-pyrimidin deposited in the form of prisms. The yield of crude material was 40 grams. It was insoluble in cold water. It crystallized from hot alcohol or water in stout prismatic crystals that melted at 190°C . to a clear oil. Analysis (Kjeldahl):

0.1811 gram of substance gave 0.02667 gram of nitrogen = 19.05 c.c. $\frac{1}{16}$ normal HCl.

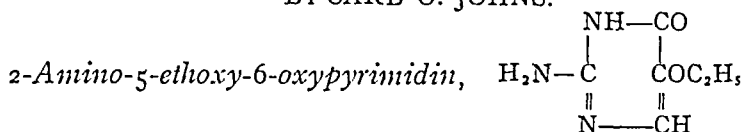
Calculated for $\text{C}_7\text{H}_{10}\text{O}_2\text{N}_2\text{S}$.

N = 15.05 per cent.

Found.

14.73 per cent.

By CARL O. JOHNS.



Eighty-two grams of guanidin carbonate were dissolved in water and combined with a solution of 150 grams of barium hydroxide. The barium carbonate was filtered off and the clear filtrate containing guanidin was added to an aqueous solution of 90 grams of the sodium salt of ethyl- α -ethoxy- β -oxyacrylate. The mixture was then allowed to stand for twenty-four hours. When it was neutralized with acetic acid no precipitate was obtained. The solution was then concentrated on the steam-bath and the base precipitated with a cold, saturated solution of mercuric chloride. This was filtered off, washed with cold water, and decomposed with hydrogen sulphide. The mercury sulphide was removed by filtration and the clear filtrate evaporated to dryness. We obtained a dark colored residue which was dissolved in water and boiled with animal charcoal to remove the color. When this solution was neutralized with ammonia, the pyrimidin base separated in the form of distorted prisms. It formed a very insoluble sulphate that crystallized from hot, dilute sulphuric acid in well developed prisms. They decomposed at 225° – 226°C . with effervescence and contained two molecules of water of crystallization:

0.8588 gram of substance lost 0.0700 gram of H_2O at 110° – 120°C .

Calculated for $(\text{C}_6\text{H}_7\text{O}_2\text{N}_3)_2 \cdot \text{H}_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$. Found.

H_2O = 8.11 per cent.

8.18 per cent.

Nitrogen determination in the hydrous salt (Kjeldahl): 0.1037 gram of salt gave 0.01960 gram of nitrogen = 14 c.c. $\frac{1}{16}$ normal HCl.

Calculated for $(\text{C}_6\text{H}_7\text{O}_2\text{N}_3)_2 \cdot \text{H}_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$. Found.

N = 18.9 per cent.

18.9 per cent.

Nitrogen determination in the anhydrous salt (Kjeldahl): 0.1255

gram of salt gave 0.02548 gram of nitrogen = 18.2 c.c. $\frac{1}{16}$ normal HCl.

Calculated for $(C_6H_5O_2N_3)_2 \cdot H_2SO_4$. Found.

N = 20.58 per cent. 20.31 per cent.

The pyrimidin base was isolated in a pure state when this sulphuric acid salt was digested in water with barium carbonate. The excess of barium carbonate and barium sulphate was removed by filtration and the filtrate evaporated to dryness. The base was obtained in the form of distorted prisms. It deposited from hot water in microscopic prisms which melted at 248° C. to a clear oil. Analysis (Kjeldahl):

0.0978 gram of substance gave 0.02618 gram of nitrogen = 18.7 c.c. $\frac{1}{16}$ normal HCl.

Calculated for $C_6H_5O_2N_3$.

N = 27.09 per cent.

Found.

26.77 per cent.

THE DETERMINATION OF SMALL QUANTITIES OF IRON.

BY W. McKIM MARRIOTT AND C. G. L. WOLF.

(From the Chemical Laboratory, Cornell University Medical College,
New York City.)

(Received for publication February 9, 1906).

There would seem to be at present no perfectly satisfactory method for the determination of such minute quantities of iron as exist in the tissues and fluids of the body and in potable waters.

The ferrocyanid method of Zangemeister,¹ although quite delicate, is open to the objection that blue colors are difficult of comparison, and the presence of free inorganic acids may themselves give rise to a distinct blue coloration with the reagent which is not due to the iron contained in the acid itself, but is probably a decomposition product of the ferrocyanid, giving rise to ferric ions.

A somewhat more delicate method is that based on the reaction which ferric salts undergo when treated with an alkaline thiocyanate.

This method has been the subject of much investigation,² as far as aqueous solutions are concerned, and it is to-day the method mostly employed in physiological research.

As usually applied, however, it is distinctly faulty when one wishes to determine accurately quantities of iron under the tenth of a milligram. It has been our object to improve this method for physiological purposes, and the following results will show to what extent this has been accomplished.

As has been repeatedly pointed out,³ the thiocyanate com-

¹ Zangemeister, *Zeitschr. f. Biol.*, xxxiii, p. 72.

² Zega, *Chem. Zeitg.*, xvii, p. 1564; Ribau, *Bull. Soc. Chim.*, (3) vi, p. 916; Krüss and Moraht, *Ber. d. deutsch. chem. Gesellsch.*, xxii, p. 2054; Liebig's *Ann. d. Chem.*, ccii, p. 260; Magnanini, *Zeitschr. f. physikal. Chem.*, viii, p. 1; Lapique, *Compt. Rend. Soc. Biol.*, 1890, p. 669.

³ *Ibid.*

pounds of iron hydrolyze in aqueous solution, giving rise to ions which are non-chromatic. Following out this line, it is found that the depth of color of the thiocyanate is dependent on the concentration of the iron, the thiocyanate, and the presence of acids and salts. In consequence of this, therefore, the color is not constant with increasing dilution, and tends to, and practically does, disappear in dilutions of no great degree. It has been claimed that the color given by ferric thiocyanates is not permanent, but fades especially when exposed to light.¹

For this reason, attempts have been made from time to time to replace the thiocyanate by other substances giving colored salts of iron.² It is on this that the method recently proposed by Pulsifer³ is based. Acetylaceton has been found to be much less delicate than the thiocyanate itself, and in addition is much more sensitive to variations in the acidity of the solution. We have also found that benzoylaceton closely resembles acetylaceton in this respect. All the compounds capable of tautomeric change, such as acetoacetic ester, benzoylacetic ester, formylphenylacetic ester, etc., and which give an enol configuration with a colored iron salt, might be used, but in all cases the aqueous solution tends to a state of equilibrium of aldol and enol modifications which may be reached slowly or quickly.⁴ With the change in concentration of the active modification a corresponding change in color takes place. The color is therefore not constant. Azoimid,⁵ which might be used on account of its colored iron salt, has not in our hands proven a success. The highly toxic character of this substance is an objection to its use.⁶

In any case, the reaction with thiocyanate, and doubtless with other substances, may be rendered more sensitive by increasing the concentration of the reagent. Ammonium thio-

¹ Pulsifer, *Journ. Amer. Chem. Soc.*, xvii, p. 967.

² Andreasch, *Ber. Akad. Wiss.*, Wien, 1879, p. 133; Böttger, *Jahresb. physikal. Ver.*, Frankfurt, 1879, p. 26; Pagliani, *Gazz. chim. ital.*, cit. *Zeitschr. f. anal. Chem.*, xviii, p. 475.

³ Pulsifer, *loc. cit.*

⁴ Wolf, *Trans. Roy. Soc. Can.*, iv., p. 91, 1898.

⁵ Dennis and Browne, *Journ. Amer. Chem. Soc.*, xxvi, p. 577.

⁶ Smith and Wolf, *Journ. Med. Research*, xii, p. 451.

cyanate is particularly suitable in this respect on account of its great solubility in water.

In order to work to extreme accuracy, it is necessary to use fairly large amounts of solution. The columns for comparison must be long, and the surface sufficiently broad to be read with facility. This requires increasingly great volumes of thiocyanate solution. It will be found, moreover, that small differences in the concentration of the thiocyanate of the standard solution and the solution to be tested will give differences in color which are quite perceptible.

Another method, which from a physico-chemical standpoint may be used to decrease the amount of hydrolysis, is to perform the reaction in a medium which does not permit of this process taking place. For this purpose, we attempted the use of ether, as did Natanson¹ many years ago. Ferric thiocyanate is much more soluble in ether than in water, and on shaking an aqueous solution containing the ferric salt with ether the color is transferred at once from the aqueous to the ethereal layer. A color, almost imperceptible in water, is rendered most distinct by shaking with ether.

This method, which promised success, unfortunately did not fulfil our expectations. The color was extremely sensitive to light in ethereal solution and was affected by numerous reagents, acids, and various salts. We were compelled to abandon it. Further, the maintenance of a constant supply of ether free from acids and peroxids was difficult.

We were therefore led to seek for other solvents which did not possess these disadvantages. The following experiments are designed to show that in acetone we have a solvent which fulfils these conditions quite perfectly.

EXPERIMENTAL.

The color comparisons were made in glass tubes 200 mm. long and 15 mm. wide, with plane glass plates cemented to one end of the tube. The tubes, which were all of exactly the same length, were filled to the top, and cover glasses slipped on in such a manner as to exclude air-bubbles. The colors in the tubes were compared in a dark box fitted with a white porcelain re-

¹ Natanson, *Liebig's Ann. d. Chem.*, cxxx, p. 246, 1864.

flector. The light, which in these determinations must be of the whitest possible, was obtained from a prism-glass window.

Standard solutions of iron were prepared by dissolving a weighed quantity of iron in hydrochloric acid, and saturating the solution with chlorine gas. The solution, evaporated to complete dryness on the water-bath, was taken up with water to which a small amount of hydrochloric acid had been added, and suitably diluted.

The standard amounts of iron were delivered from a burette graduated to 0.05 c.c. and accurately read to 0.01 c.c. In order to keep the dilution constant, the mixtures were always made up in graduated glass-stoppered cylinders to a definite volume, and a portion transferred to the comparison tubes, which as stated above were all of the same length.

The Influence of Concentration of Ammonium Thiocyanate in Aqueous Solution.

Ferric chlorid solution No. 2. One c.c. contains 0.0001 gram Fe. Volumes made up to 50 c.c.

Fe Solution. c.c.	NH ₄ CNS, 10 per cent. 1 c.c.	NH ₄ CNS, 25 per cent. 1 c.c.
1.0	Rose	Rose
2.0	Red	Blood red.*
3.0	Blood red*	Dark red
4.0	Dark	Dark red

Solutions marked * have the same color.

Ferric chlorid solution No. 4. One c.c. contains 0.00001 gram Fe.

Fe Solution c.c.	NH ₄ CNS, 10 per cent. 1 c.c.	NH ₄ CNS, 25 per cent. 1 c.c.
1.0	0	0
2.0	Light straw	Light straw
3.0	Light red	Red*
4.0	Red*	Darker red

Solutions marked * have the same color.

These results show that not only is the variation in the intensity of the color due to the concentration of the thiocyanate, but that

the concentration of the iron itself interferes as a second factor.

The effect of acids on ferric thiocyanate proceeds in two directions. With 1 c.c. of normal hydrochloric acid, a slight darkening in color is produced with 0.00005 gram of iron. With 5 c.c. the solution is lighter than with 1 c. c., and with 10 c.c. the color is practically the same. Acetic acid produces the same effect. Larger amounts of this acid are necessary to change the color. This corresponds with the slight dissociation of the acid. Potassium chlorid and ammonium chlorid are alike in their behavior. A color produced with one-twentieth of a milligram is discharged in the presence of 5 c.c. of a 10 per cent. solution of these salts.

The Behavior of Ferric Thiocyanate in Ethereal Solution.

A definite amount of iron solution was measured into a 50 c.c. stoppered cylinder. One c.c. of ammonium thiocyanate solution was added. Water was added to 10 c.c. and ether to 50 c.c. The mixture was shaken, and the ether transferred to a separating funnel, and subsequently to comparison tubes.

Using 3 c.c. of iron solution No. 3 ($=0.00003$ gram Fe) with 1 c.c. of 25 per cent. ammonium thiocyanate, the color was the same as that with 3.2 c.c. of the iron solution and 1 c.c. of the 10 per cent. thiocyanate. It was evident that here, also, the influence of the thiocyanate was marked, although not so much as in aqueous solutions, and that except for the increased delicacy the method would possess no advantage.

With hydrochloric acid, with acetic acid, and with ammonium and potassium chlorids, the effects were the same as those noted with the aqueous solutions. The color, moreover, was not constant when exposed to light. A standard solution faded, when exposed in a window, to 50 per cent. of its original intensity in thirty minutes.

The Use of Aceton as a Solvent.

The aceton used was Kahlbaum's commercial aceton. It was redistilled from calcium chlorid, using a long fractionating column. It had a boiling point, uncorrected, of 56.5° – 57.5° C.

In a preliminary test, it was found that a solution of iron and ammonium thiocyanate diluted with aceton had a much darker color than the same solution made up with water.

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The Influence of Neutral Salts on the Reaction in Aceton.

In determining the effect of various salts on the color produced in aceton, it was found that ammonium chlorid and potassium chlorid diminished the intensity of the color when 1 c.c. of normal ammonium chlorid in 15 c.c. of water was contained in the 50 c.c. of iron solution to which the iron was finally diluted.

Hydrochloric acid and acetic acid in small amounts slightly increase the color.

The effect of potassium and ammonium chlorid may be eliminated by the use of more ammonium thiocyanate, as is shown below.

In the comparison tube of a Gallenkamp colorimeter was placed a solution made up as follows: Four c.c. of $\frac{N}{1000}$ iron, 2 c.c. of normal ammonium thiocyanate, water to 15 c.c., and aceton to 50 c.c. In the prism of the colorimeter were put solutions made up as indicated in the table. The solutions all contained the same concentrations of water and aceton. Equal readings of the colorimeter indicate equal intensities of color.

$\frac{N}{1000}$ Fe	N NH ₄ CNS	N KCl	N HCl	Reading	N NH ₄ CNS	N KCl	N HCl	Reading
0.5	1	0	0	86	1	1	0	98
0.5	2	0	0	78	2	1	0	80
0.5	5	0	0	73	5	1	0	73
0.5	10	0	0	73	10	1	0	73

This experiment shows that with a concentration of 5 c.c. of normal ammonium thiocyanate the effect of 1 c.c. of potassium chlorid is done away with. The same amount of thiocyanate is nearly sufficient to counteract the effect of double the amount of potassium chlorid.

$\frac{N}{1000}$ Fe	N NH ₄ CNS	N KCl	Reading
0.5	1	2	103
0.5	2	2	86
0.5	5	2	76
0.5	10	2	73

The color in aceton is remarkably permanent when exposed to bright or diffused light. A standard solution in aceton gave a duplicate reading on comparison with a freshly prepared solution after having stood three days in a window.

It has been stated¹ that the presence of phosphates renders the reaction with thiocyanates unreliable. As this is a matter of importance in biological analysis, where solutions not infrequently contain notable quantities of the salts of phosphoric acid, we have investigated the matter and find that phosphates in solution do interfere with the reaction. This may, however, be completely avoided by increasing the concentration of the hydrochloric acid used in acidifying the solution, as the following experiment shows:

Solution 1, containing 4 c.c. of 10 per cent. disodium phosphate, 0.00001 gm. Fe, 10 c.c. N HCl, 10 c.c. N NH₄CNS, and acetone to 50 c.c.

Solution 2, containing the same components with the exception of the phosphate solution.

In the comparison tubes the colors are identical.

Among other salts which we have tried, and which are said to affect the color,² are the borates, tartrates, and oxalates and the salts of mercury. The last named, as is well known, are used to differentiate the meconates from the thiocyanates.

In the comparison 0.3 gram of boric acid produced no color change. One c.c. of glacial acetic acid produced an almost imperceptible darkening. Two-tenths of a gram of oxalic acid completely removed the color, and smaller quantities had a marked effect. Three-tenths of a gram of mercuric chlorid caused a slight decrease in color. The effect of 0.1 gram was almost negligible.

The following substances to the extent of 0.3 gram produced no change in color: aluminium sulfate, barium nitrate, manganese sulfate, zinc sulfate. In the case of aluminium and zinc, the salts (Kahlbaum's "K") were found to contain iron and this was allowed for.

The Delicacy of the Reaction in the Three Media.

Experiments have been made to compare the delicacy of the reactions as far as possible under equal conditions.

With aqueous solutions containing 0.25 gram of ammonium thiocyanate in 50 c.c., 0.00002 gram of iron gave a pale straw

¹ Moore, *Chem. News.*, liii, p. 289.

² Prescott and Johnson, *Qual. Anal.*, 1903, p. 155.

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color in the comparison tube. A faint pink color is first reached when the solution contains 0.0001 gram of iron.

With ether 0.000001 gram of iron can be detected when 10 c.c. of water containing this amount of iron and 0.25 gram of ammonium thiocyanate are shaken with 40 c.c. of ether.

In the same volume of solution 0.000005 gram of iron is readily detectable when 25 c.c. of an aqueous solution, containing acid and ammonium thiocyanate, are diluted with 40 c.c. of acetone. The difference between 0.000025 gram of iron and 0.000027 is very apparent, as is also the difference between 0.000002 and 0.000003 gram of iron.

In the determination of iron in an unknown solution, two methods are available.

In the first, the substance having been brought suitably into solution (free from oxalates), 2 c.c. of 5N hydrochloric acid are added, and the solution made up to 15 c.c. with water in a 50 c.c. stoppered cylinder. Ten c.c. of N ammonium thiocyanate are added, and acetone to 50 c.c. In a second cylinder are placed 2 c.c. of 5N hydrochloric acid, and 0.5 to 2.0 c.c. of a standard solution containing 0.00001 gram of iron per c.c., 10 c.c. of N ammonium thiocyanate, and acetone to 50 c.c. The contents of the two tubes are then transferred to the comparison tubes, and the colors noted. The lighter colored solution is returned to the cylinder and ferric chloride added till the two colors are identical.

As the volumes are practically the same, the increased hydrolysis, owing to the water in the additional iron solution added, being negligible, the two solutions contain the same amount of iron. A simple calculation then gives the amount of iron in the unknown solution.

A better method consists in the use of a diluting solution. As has been seen, even with acetone, the color perceptibly changes on dilution, and this holds good also with acids and for thiocyanate.

By the use, however, of an acetone solution containing hydrochloric acid and ammonium thiocyanate this difficulty is obviated. The solution has the following composition:

Acetone — 100 c.c., 5N hydrochloric acid — 8 c.c., N ammonium thiocyanate — 40 c.c., water to 200 c.c.

If a solution made up according to the previous method be diluted with the foregoing diluent the intensity of color will be exactly inverse to the dilution.

The following determinations were made to test the accuracy of the method:

Cylinder I.		Cylinder II.			
Volume	Fe added	Volume	Fe added	Fe found	Error
76	0.000015	50	0.000010	0.0000099	0.0000001
68	0.000015	50	0.000011	0.0000110	0.0000000
63	0.000015	50	0.000012	0.0000119	0.0000001
59	0.000015	50	0.000013	0.0000127	0.0000003
54	0.000015	50	0.000014	0.0000139	0.0000001
50	0.000015	50	0.000015	0.0000150	0.0000000

The method has been applied to the determination of the iron contained in an S. and S. filter paper No. 590, 9 cm. A single paper was sufficient for the determination. The paper was ignited, and brought into solution by the method given below. Each paper in a given package was found to contain 0.0000035 gram of iron when folded with platinum tongs. When folded with the fingers the amount was increased to 0.000004 gram, due to the iron taken up from contact with the fingers. It will be seen that the method requires scrupulous care on account of its delicacy.

The Determination of Iron in Tissues and Organic Fluids.

In order to test the method with fluids containing proteins the following experiment was performed:

In a platinum dish were placed 0.2 c.c. of a concentrated solution of egg albumin. To this was added a standard iron solution containing 0.000025 gram of iron. The solution was evaporated and ignited till the residue was nearly white. One-tenth of a gram of potassium hydrogen sulfate (Kahlbaum's "K") was added. The ignition was repeated till the residue was quite white, and all sulfuric acid fumes had been driven off. After cooling, the residue was dissolved in 2 c.c. of warm water containing 2 c.c. of 5N hydrochloric acid. The solution was rinsed into a cylinder till the volume was 15 c.c. Ten c.c. of N ammonium thiocyanate were added and the mixture made up to 50 c.c. with acetone.

In a second cylinder the results of the same operation were placed, omitting the albumin solution.

In a third, the iron solution was directly added without the previous fusion with acid potassium sulfate. On comparison, all the solutions had identically the same color. A control made, using the same amounts of albumin and potassium hydrogen sulfate showed the absence of iron in the amounts used.

*The Method Applied to the Estimation of the Amount of Iron in the Blood.*¹

With a capillary pipette 0.025 c.c. of blood is drawn up. The blood is expelled into a platinum dish and the remaining blood washed out from the pipette with several washings with small amounts of distilled water. The solution is evaporated to dryness, ignited, fused with 0.1 gram of acid potassium sulfate, heated to redness till all fumes have been driven off, and the residue has a faint yellow color. It is then dissolved out with 2 c.c. of warm 5N hydrochloric acid, rinsed into a cylinder with warm water, and compared in the usual manner. The following are some results with dogs' blood taken from the marginal artery of the ear. The amounts of iron found under certain experimental conditions varied from 0.0046 per cent. to 0.0054 per cent.²

For tissues, the method is similar. A weighed amount of the substance is ignited in a platinum dish, and fused with acid potassium sulfate.

The Determination of Iron in Inorganic Substances.

On account of the extreme delicacy of this method the amount of iron can be determined in many cases with considerably less than a gram of the substance, by simply dissolving the substance in water, and making up in the usual manner. For liquids, acids, etc., less than 1 c.c. will generally be sufficient. If very small amounts of iron are to be determined, the solutions may be concentrated by evaporation. In examining many salts for which more than 0.5 gram is required, it will be

¹ Lapicque, *Compt. Rend. Soc. Biol.*, 1889, p. 167; 1895, p. 59; Jolles, *Monatsh. f. Chem.*, xvii, p. 677; Mackie, *Lancet*, 1898, p. 219; Jolles, *Deutsch. med. Woch.*, 1898, No. 7; *Wien. klin. Woch.*, 1899, No. 14; *Centralbl. f. inn. Med.*, xx, p. 681; *Munch. med. Woch.*, 1901, p. 342.

² Abderhalden, *Zeitschr. f. physiol. Chem.*, xxv, p. 65.

found that owing to their sparing solubility a precipitate will be given on the addition of the aceton. The salt is therefore dissolved in water with the addition of hydrochloric acid. To the solution three volumes of aceton are added. This will precipitate the greater part of the salt, holding the ferric chlorid in solution. The solution is filtered through a small filter paper previously extracted with aceton. It is then evaporated to dryness in a platinum dish, the residue taken up with water and estimated colorimetrically.

*The Method Applied to Urine.*¹

Two general methods have been tried with the urine. In the first, the urine was evaporated with a small amount of sulfuric acid, and fused with acid potassium sulfate. The second method was that proposed by Zickgraf,² which consists in precipitating the iron in the urine as an albuminate. Neumann's method³ has not in our hands proven suitable. By none of these methods have we found anything like the amounts of iron which these investigators have stated are present in the urine. It is noteworthy that in some of the cases the correction for iron in the reagents employed was almost as much as the total amount of iron in the specimen of urine under examination.⁴

Should there be such amounts of iron in the urine as have been stated, 25 c.c. of the fluid should by this method be ample for its determination, yet using these amounts of urine we have as yet been unable to detect the most minute quantities.

The matter is still under investigation.

¹ Zickgraf, *Zeitschr. f. anal. Chem.*, xli, p. 488; Neumann, *Zeitschr. f. physiol. Chem.*, xxxvii, p. 115.

² Zickgraf, *loc. cit.*

³ Neumann, *loc. cit.*

⁴ Zickgraf, *loc. cit.*, pp. 491, 493.

ON THE CHEMISTRY OF BACILLUS COLI COMMUNIS.

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For the past few years, large incubating tanks have been in use in this laboratory for raising pure cultures of bacteria upon solid media. Each tank provides twenty square feet of growing surface, and yields under favorable conditions from thirty to fifty grams of air-dried cell substance. Thus it is possible to obtain sufficient material to make a systematic study of the chemical composition of the bacterial cell. It is proposed in this paper to give a review of the literature upon this subject, a *résumé* of some preliminary work upon the cell substance of *Bacillus coli communis*, a more detailed account of a toxic product split off by very dilute acid, and also of the preparation and purification of lysin from the same germ.

LITERATURE.

In the absence of data in regard to the colon bacillus, a study was made of the literature upon the chemical composition of other forms of bacteria, although it is not safe to assume that whatever has been ascertained concerning one species is true of another. Indeed, Nencki thinks there are greater differences in the chemical composition of nearly related forms among bacteria than among other orders of either plants or animals.

A large amount of work is reported upon the extracellular activity or the changes produced in the culture medium; indeed some of this study antedates the discovery of bacteria. Again, the application of the methods of physical chemistry to the various problems of bacterial chemistry opens a most profitable field of investigation. But both of these topics are beyond the scope of this review, except in such cases as have bearing upon the chemistry of the cell or of intracellular products. However,

it is often very difficult to tell from the data given whether the products studied are intracellular or extracellular. This is especially true of work upon bacterial poisons.

ANALYSES. A number of the earlier observers have grown bacteria on solid media, removed the growth, and determined the moisture and ash, also the amount of substance soluble in alcohol and ether. The results are given in Table I; Table II includes a number of elementary analyses.

TABLE I.
MOISTURE, ASH, AND SUBSTANCE SOLUBLE IN ALCOHOL AND ETHER.

Germ.	Observer.	Medium.	H ₂ O. per cent.	Ash in dry subs. per cent.	Ash in moist subs. per cent.	Extract per cent.
Putrefying bac.	Nencki	Gelatin	83.42	4.72*		6.04
Pneumo. bac.	Brieger	"	84.20	30.24		1.74
B. anthracis	Dyrmont	"	85.44			
B. prodigiosus	Kappes	Agar	85.45	13.47		4.83
B. xerosis		"	84.93	9.52		8.06
B. tuberculosis	Hammerschlag	Gly. agar	85.00	8.		3.84
B. prodigiosus	Cramer	Potato at 33°	75.83	9.31	2.26	
Do.	"	" 15°	80.02	12.52	2.63	
Do. 4 days old	"	"	79.56	11.33	2.41	
Do. 14 days old	"	"	82.55	13.77	2.38	

* Per cent. of dry substance after extraction with alcohol.

Kresling found that *Bacillus tuberculosis*, air-dried, lost 3.93 per cent. by drying at 100° to 110°C., 3.018 per cent. by drying three months in a desiccator over sulfuric acid.

TABLE II.
ELEMENTARY ANALYSES.

Germ.	Observer.	Medium.	Carbon.	Hydro- gen.	Nitro- gen.	Sul- fur.	Phos- phor.
B. tuberculosis	Hammerschlag	Gly. agar	51.62	8.07	9.09		
"	Levene	Beef broth	55.58	8.46	9.39	1.39	0.55
"		Mannite syn.	47.41	7.05	7.91	0.25	0.61
"*	DeSchweinitz, Dorset	Beef broth	60.12	9.15	7.27		0.81
"	"	"	62.08	7.39	8.04		0.81
"	"	Artificial	62.16	9.10	8.94	0.22	0.61
Putrefying bac.	Nencki	"	53.82†	7.76	14.02		
B. diphtheriæ	Dzierzowski	Peptone	48.87‡	8.61	11.17	1.38	0.61
B. anthracis	Dyrmont	Gelatin					
spores	"	"	52.1	6.82	16.2		
threads	"	"			6.8		
Water bac. No. 28	Nishimura	Potato	50.08	6.75	11.05	1.02	
Erythema nodosum	Bovet	"	48.13	7.01	11.60		
B. Mallei	DeSchweinitz, Dorset	Beef broth	41.81	5.89	14.05	0.99	1.11
Swine plague	"	Peptone Do.	44.57	7.20	11.81		

* Calculated per cent. of dry substance.

† Free from ash and fat.

‡ After extraction with alcohol and ether.

On comparing these figures, one notices not only the diversity between different species, but the variations in different cultures of the same germ. Cramer, after an exhaustive study of the

subject, concludes that there is no typical value for moisture and ash; both depending upon conditions of growth.

As regards the ash, Cramer, in some very interesting work on the cholera bacillus, finds that in this germ it depends within certain limits, both qualitatively and quantitatively, upon the culture medium. For instance, by using media rich in phosphates or chlorids, the amount of ash can be increased to nearly 30 per cent. of the dry substance. He also notes that the carbon and hydrogen are fairly constant for all species, not far from 51 per cent. and 7 per cent. respectively; while the nitrogen may vary widely, from 5.34 per cent. to 11.15 per cent. in *subtilis* as found by Vincenzi, and to 16.2 per cent. in anthrax as reported by Dyrmont. His observations of the effect of increasing the peptone in the medium, and of using grape sugar, show that bacteria adapt themselves to the culture medium, especially as regards their proteid constituent. Thus, comparable results can be obtained only from cultures of similar species, at the same stage of development and grown under like conditions.

Lyons has observed a certain relation between the carbohydrate formation and the carbohydrate in the medium. Thus, with increase of grape sugar in the medium, he found in the germ less proteid and more substance extracted by alcohol and ether.

Rodet calls attention to variations in chemical properties in the same species due to variations in the media, in temperature, light, etc. Variations in the indol reaction of the colon bacillus afford a familiar example. He states further that the pigment of *Bacillus pyocyaneus* depends upon the amount of oxygen, and in albumin cultures is green, in peptone cultures blue; that the virulence of the cholera vibrio, which has been diminished by laboratory culture, can be increased by growth on gelatin containing phosphate or a trace of iron.

Lepierre finds α -glucoproteins having the general formula $C_nH_{2n}N_2O_4$ ($n=6-11$) especially adapted to furnish nitrogen to microorganisms. Of forty-five different organisms studied, most used any glucoprotein; but diphtheria, tetanus, and some others preferred those containing eight or nine atoms of carbon; while some, tuberculosis for example, preferred those with

higher carbon content. However it is not surprising that organisms sufficiently sensitive to be used in separating stereoisomers should respond to slight changes in external conditions.

PROTEIDS. The earliest separation of proteid from bacteria was the isolation of mykoprotein from putrefying bacteria by Nencki and Schaffer in 1879. Later Nencki attempted to prepare mykoprotein from *Bacillus anthracis*, but obtained a new body, which he called anthrax protein. Brieger found in Friedländer's pneumococcus a proteid which contained less nitrogen than does mykoprotein. Lewith reports that Hellmich obtained from hay bacilli a substance with the properties of a globulin. Buchner obtained proteids from many different species of bacteria by extracting with water, with dilute alkalis, and also with 40 or 50 per cent. glycerin. Krehl and Matthes digested bacteria of various strains with pepsin, and isolated deuteroalbumose according to Neumeister's method. Brieger and Fränkel, Wasserman and Proskauer, and Dzierzgowski and Rekowski, all have reported toxalbumins from diphtheria cultures. Murillo also reported a toxic proteid from the same germ. Hammerschlag obtained from tubercle bacilli a proteid which he believed to be the source of the acid-fast character; but as the germs were also extracted with alcohol and ether, his reasoning would apply as well to the fatty constituents as to the proteid. Von Hoffman reported six proteids, and Weyl reported a toxomucin from the tubercle bacillus. Nitta obtained from crude tuberculin an albumose which acts like tuberculin, but is five times as active. As the specific action is destroyed neither by sodium nitrite, formaldehyde, nor by hydroxylamin, he concludes that it is not due to a labile amido, aldehyde, or ketone group. From *Bacillus tuberculosis* Ruppel obtained tuberculosamin, a body having the properties of a protamin.

NUCLEIN AND RELATED COMPOUNDS. From their action toward basic anilin dyes, Dreyfuss argued the presence of nuclein in all bacteria; Gottstein advanced as additional proof, their behavior with hydrogen peroxid, and the presence of phosphorus. Vandevelde reported finding nuclein in *Bacillus subtilis*. Dietrich and Liebermeister found microchemical evidence that granules in old bacilli contained nuclein. Nishimura separated nuclein bases from a water bacillus grown on potato. Lustig and

Galeotti reported a nucleoproteid from the pest bacillus. From an organism resembling *Bacillus ranicidus*, Galeotti obtained a nucleoproteid having 11.99–12.21 per cent. of nitrogen, and 0.94–1.16 per cent. of phosphorus. From anthrax bacilli, Casagrandi, Galeotti, Paladino, and Tiberti prepared nucleoproteids for immunity experiments. Ruzicka regarded nuclein as the chief constituent of this germ. Aronson obtained from the bacillus of diphtheria, one product which gave proteid tests, and upon decomposition yielded xanthin bases, pentose, and albumin. A second substance from the same bacillus yielded xanthin bases, pentose, and phosphate. Blandini reported nuclein and nucleoalbumin from the typhoid bacillus. Enea obtained from both *typhosus* and *subtilis* a toxic nuclein. From the bases and phosphorus in extracts of *Sarcina lutea*, Wheeler found evidence of a nuclein body in that germ. From several different germs Iwanoff reported nucleoproteids which gave the biuret reaction, and contained about 16 per cent. of nitrogen, 2 per cent. of phosphorus, 2 per cent. of sulfur, and 2 or 3 per cent. of ash. Certain kernels found in bacteria, Meyer regards as reserve material consisting of nucleoproteid with a relatively large amount of nucleic acid derivatives.

Several investigators have found nucleoproteids in *Bacillus tuberculosis*. Klebs reported a nuclein containing 8–9 per cent. of phosphorus; de Schweinitz a nucleoalbumin; while by a pressure of 400–500 atmospheres upon the moist germs, Hahn obtained a relatively large amount of nucleoalbumin. Ruppel separated a nucleic acid containing 9.42 per cent. of phosphorus, which he designated as tuberculinic acid. Levene reported from the same species, three proteids, nuclein, tuberculinic acid, and crystals which he regarded as a mixture of thymin and uracil, also cytosin. Kutscher, Wahlen, and Trudeau, Baldwin and Kinghorn also reported nucleo-compounds from this germ. Kitajima described several different toxic substances, all of which contain tuberculinic acid (*Tuberkelthyminsäure*).

Wheeler has analyzed a phosphorized glycoproteid from *Bacillus typhosus* with the following percentage composition: C–48.27, H–6.28, N–10.92, S–1.45, P–0.403, and a trace of iron. Vaughan considers the colon germ as a chemical compound, a glyconucleoproteid. Lepierre and Charrin and Desgrez

report mucin from different germs; while Rettger states that he has found it as a product of many species of bacteria, independent of the presence of carbohydrate in the culture medium.

CARBOHYDRATES. Scheibler, and Smith and Steel reported gums from the viscous growth of bacteria in sugar juice. Schar-dinger, Ward and Green, and Kramer obtained carbohydrates from bacterial slime. From *Bacillus subtilis* Dreyfuss obtained a substance which reduced Fehling's solution, and from which he obtained glucosazone crystals. This he regarded as evidence of cellulose, but as Vincenzi failed to find it in the same germ by other methods, Dreyfuss's results were probably due to some other carbohydrate. Hemicellulose was found by Nishimura in a water bacillus, in *prodigiosus*, and in *Staphylococcus pyogenes*. De Schweinitz and Dorset found evidence of a small amount of cellulose in the tubercle bacillus, but not in the bacillus of glanders; Hammerschlag also reported cellulose in the tubercle germ; and Brown obtained it from the membrane of *Bacterium xylinum*. Wheeler found in yellow sarcine two carbohydrates, but no evidence of cellulose.

Both Bendix and Aronson obtained pentose from bacteria. Iwanoff, Emmerling, Helbing, and Bulloch reported chitin from germ substance. Levene has obtained glycogen from *Bacillus tuberculosis*.

FAT, WAX, ETC. In the earlier investigations of the composition of bacteria, alcoholic and ethereal extracts were often assumed to be fats without further investigation. Cramer, Meyer, and Klebs, all isolated fatty substances from germs. Nishimura found various fatty acids in a water bacillus, and isolated 0.68 per cent. of the dry germ substance as lecithin.

From the tubercle bacillus Hammerschlag reports obtaining fatty acids which melt at 63°C ; de Schweinitz and Dorset have isolated several acids, one of which was crystalline, melting at $161\text{--}164^{\circ}\text{C}$., and having the composition corresponding to the formula, $\text{C}_7\text{H}_{10}\text{O}_4$. Ruppel and Aronson have obtained wax as well as fat; that is, esters of acid and high alcohol, as well as esters of acid and glycerin. Levene finds that this wax melts between 55° and 60°C ., its analysis corresponds to the empirical formula $\text{C}_{12}\text{H}_{24}\text{O}_3$, and it is not saponified by ordinary methods.

Bulloch and MacLeod have isolated a pure alcohol which retained stain after immersion for eight days in 25 per cent. sulfuric acid. Klein finds that the young bacilli are not acid-fast, but thinks that property due to chemical substances which they produce in later stages of growth; while Marmorek regards the young germs, containing very little wax and fat, as the ones concerned in the tuberculin reaction. Dorset and Emery also refer the characteristic staining properties of this germ to higher alcohols of the aliphatic series. Kresling reports 38.95 per cent. of fat, 0.16 per cent. of lecithin, and some cholesterin from the tubercle bacillus. MacDonald calls attention to the resemblances between wool fat, hydrous cholesterin, and the fat of this germ.

Beebe and Buxton found the pellicle on a growth of *Bacillus pyocyaneus* to consist of masses of bacteria and bundles of fatty crystals. Lipochromes and other pigments have been reported as integral parts of the bacterial cell. Thus, Jordan obtained a blue pigment from *B. pyocyaneus*. Detweiler separated pigments from *B. violaceus* and from *prodigiosus*, and gives a summary of the literature of bacterial pigments.

CELL PLASMA. Hahn mixed bacteria with sand and subjected them to a pressure of 400-500 atmospheres. He thus obtained a clear liquid, which contains much coagulable albumin, shows reducing action, gives the ordinary proteid reactions, and undergoes autodigestion less readily than yeast plasma. Macfadyen and Rowland obtained cell plasma by rubbing up dry germs in a mortar submerged in liquid air, moistening the mass with physiological salt solution, and centrifugating. This gives an opalescent solution of the intracellular constituents. One cubic centimeter from typhoid germs, dried at 100°C., gave 0.07174 gram of substance, of which 0.027 gram was fixed ash. The residue contained 6.914 per cent. of nitrogen and 37.64 per cent. of ash. The plasma of typhoid, streptococcus, and several other bacteria was found highly toxic. Various species of bacteria were kept for six months immersed in liquid air without impairing their vitality. Bassenger and Mayer also froze and pulverized germs, getting a plasma which gave proteid reactions, but gave negative results with Bial's pentose reagent and Fehling's solution. Maasen found plasma to show

reducing action, and thinks there are two reducing substances in the cell, which are very labile, resembling ferments.

ENZYMES. Emmerich and Low find that pathogenic bacteria produce proteolytic enzymes which dissolve the nucleoproteid of the bacterial cell, and for which they suggest the name nuclease. The nucleases give oxygen with hydrogen peroxid, dissolve thymol-gelatin, egg albumin, and fibrin. Some render bacterial poisons non-toxic. In dry form they are quite stable; a solution can be made more stable by the addition of dextrin. They unite with albumins to form complex bodies of high molecular weight which are said to be very efficacious in conferring immunity. Pyocyanase, a nuclease from *Bacillus pyocyaneus*, is a yellowish green, amorphous powder, dissolving in water to form an alkaline solution. Ferrocyanic acid does not precipitate it, nor does it give the Millon and biuret tests. It is stable at 100° C., and is said to resemble trypsin. Both Dietrich and Petrie think the effects ascribed to pyocyanase to be due to other causes; to alkalinity, to action of poisonous organic bases, to starvation, or to change in osmotic pressure. Fermi also believes that bacterial enzymes can not digest the bacterial cell.

Rogers, Malfitano, Abbot and Gildersleeve, Savage, Grau, Jones, Eijkman, Levy and Pfersdorff, and Mavrojannis, all find evidence of enzymes in bacteria, some precipitating casein, some liquefying gelatin, others, hemolytic, amylolytic, proteolytic; but they do not give chemical data.

Rettger grew bacteria of various kinds upon cooked potato, scraped off the growth, mixed it with physiological salt solution and either toluol or chloroform. The mixture was tested from day to day with alkali and copper sulfate, the intensity of the color showing the progress of digestion. With the colon germ autolysis was slow; while with prodigiosus, autolysis was complete in 10 or 12 days, no proteid being left. Among the decomposition products were considerable leucin, little tyrosin, basic products considerable in amount but not enough for identification, and phosphoric acid. Young and freshly prepared mixtures were free from the odors characteristic of autolysis, and gave little leucin or other bases. With *B. pyocyaneus* autolysis was even more rapid, the proteid completely disappearing in four or five days. The author believes that auto-proteolysis goes on in

living cultures, and is a cause of irregularities in staining. He also suggests that diphtheria toxin may be intracellular, and may be liberated in this way. He finds autolysins sensitive to antiseptics, toluol being more active than chloroform.

TOXINS. Brieger obtained from tetanus cultures tetanin, $C_{13}H_{30}N_2O_4$, and tetanotoxin, $C_5H_{11}N$, well characterized poisonous compounds giving crystalline salts. He also reported typhotoxin, $C_7H_{17}NO_2$. Recently, however, little attention has been paid to these compounds, and Vaughan and Novy say that they are not to be regarded as the true toxins of these germs. Bertarelli found the cell substance of *Bacillus prodigiosus* fatal to various animals, while the soluble products were only slightly toxic. J. W. Vaughan reports an endotoxin from the anthrax bacillus, and gives an abstract of the literature upon the subject. Kolle gives a good discussion of the distinctions between extracellular toxins and endotoxins. He, Yersin, Calmette and Borrel, Pfeiffer and Dieudonné, all agree that the toxin of pest is intracellular, while Markl, and Kossel and Overbeck regard it as extracellular. Todd thinks the bacillus of dysentery contains endotoxin, as the toxin is found in young cells and in old cultures. Lüdke holds that the poisons found in filtrates from typhoid and dysentery cultures are endotoxins, set free by autolysis. Conradi reports that these toxins are weakened by antiseptic autolysis, and even by aseptic autolysis if continued longer than forty-eight hours. From streptococcus both Aronson and Bonome report an endotoxin, which Marmorek says belongs to the diastase group. Simon finds in the same germ a relatively weak endotoxin, and a much stronger extracellular poison, the secretion of these two being independent of each other. Kossel reports a toxin formed in the cell, while Gelston finds both extracellular and intracellular poisons in the diphtheria bacillus, cultures giving the most powerful extracellular toxins not giving the most toxic germ substance. Neisser and Wechsberg report two poisons from staphylococcus, having different haptophore and toxophore groups. From the tubercle bacillus Maragliano extracts a toxin by means of water, and Sciallero one by means of olive oil and ether. Rodet, Lagriffoul, and Wahby after several years of investigation, conclude that typhoid cultures are more toxic than the germ, that the toxin is not a necessary constituent

of the germ, is not an endotoxin, but a secretion. Kiessling gives a *résumé* of work upon the toxicity of the colon germ. Tichomiroff finds that nucleic acid precipitates tetanus and diphtheria toxins. Murillo reports toxin in diphtheria protoplasm, and that the toxicity of diphtheria cultures has two maxima, one in the first week and another in the third week. Charin separates from the toxin of *B. pyocyaneus*, a volatile constituent, another soluble, and a third insoluble in alcohol, each having a different physiological action.

Brieger and Boer report toxins from tetanus and diphtheria which do not give proteid and peptone tests, but seem nearly related to acids. Baldwin and Levene, however, report that both these toxins are digested by proteolytic enzymes, and so infer their proteid nature. Since they found tuberculin destroyed by trypsin but merely weakened by pepsin, they argue that it is related to nucleoproteids. Kinghorn succeeded later in rendering tuberculin inactive by six to ten days' peptic digestion. Belfanti thinks diphtheria toxin a true nuclein. Hayashi has obtained a preparation which he regards as a double compound of tetanus toxin and basic zinc carbonate. He considers the toxin a proteid, possibly a primary albumose. Brieger and Boer report that certain zinc salts precipitate tetanus and diphtheria toxin quantitatively, but that the compound contains no trace of albumin or peptone. By similar methods Brieger and Kempner obtain toxin from *B. botulinus*. Baudran subjected various toxins to the action of calcium permanganate, and found the products similar to those formed from strychnin under the same conditions. Brieger reports a toxin from *staphylococcus* which gave the alkaloidal reactions. Gautier suggests that the toxins usually consist of an alkaloidal substance joined to a very active nitrogenous body. Baldi thinks that in diphtheria cultures toxin replaces sulfur in the proteid molecule. Austin obtained toxin from typhoid and from diphtheria germs, and suggests that these toxins may be united to albumose much as iron or phosphorus is bound in caseose or casein. Auclair extracted the cell substance of various species of bacteria with ether or chloroform, and obtained products which showed the toxic action of the germs from which they came. He concludes that a study of the toxins is more important than the life history of the germ,

and that these extracts may serve for the identification and differentiation of germs, and as possible producers of immunity. Wechsberg regards the toxins as receptors of bacteria, a part of the cell, and finds that immunization *in vitro* increases the throwing off of these side chains; by growing diphtheria germs in bouillon containing antitoxic serum he increased the toxicity tenfold. Zangger calls attention to the great variations in their chemical properties shown by the toxins; most, however, are colloids and basic, some lose their toxicity by the action of solvents. By using a dialyser in which the tension of the membrane can be varied, van Calcar finds evidence that diphtheria toxin has a lower molecular weight than toxon, and toxon than peptone. Römer ascribes van Calcar's results to the filling of the pores of the membrane, although he had reached similar conclusions by quite different methods. Hailer and others agree that diphtheria toxin has a lower molecular weight than antitoxin. Biltz, Much, and Siebert find in the neutralization of toxin, that the toxin is first bound to the antibody by adsorption, following the general rules of colloids, and then this compound decomposes more rapidly than the free toxin. Gay thinks that toxins enter into direct combination with the protoplasm of the cell. Werner finds oxygen necessary to the germination of many bacilli, while on the other hand it destroys the toxin secreted. This perhaps explains the lack of virulence in artificial cultures.

By means of an alcoholic solution of sodium hydrate, Wheeler has split off from the colon bacillus an exceedingly virulent poison, which is soluble in water and in alcohol, gives the ordinary proteid color reactions, but does not yield a reducing carbohydrate. She finds no evidence that it is alkaloidal. It is broken up by concentrated acid, giving a toxic crystalline compound. Vaughan regards the poison obtained by this method the specific toxin of the colon germ, containing both haptophore and toxophore groups. He gives chemical and physiological reasons for suspecting the toxophore group to be a neurin-like body. In this connection it is interesting to note that Kyes and Sachs find a lecithin group necessary to the hemolytic action of cobra venom, and Kyes suggests that cholin may be the toxophore group of lecithin. Wolff thinks endotoxins identical with the albu-

minous substance of the cell, and their action that of any other foreign proteid. Foreign proteids do not cause the formation of antibodies, and this is the great distinction between endotoxins and toxins. Vaughan thinks the formation of endotoxins a synthetic process, not essential to the life of the cell, nor are they the sole constituents of the cell plasma.

Perhaps no recent chemical problem has attracted more widespread attention than the application of the methods of physical chemistry to the problems of physiological chemistry, and especially to the neutralization of toxins by antitoxins. Arrhenius, Madsen, and others find experimental evidence that it follows the law of mass action; Ehrlich, Sachs, Nernst, and others hold that the action is not reversible, and is much more complicated.

IMMUNE BODIES. Agglutinums, precipitums, and hemolysins have been split off from the bacterial cell, but their study has been physiological rather than chemical. Pick, who gives an extensive bibliography, has made an exhaustive study of the substances from typhoid germs which react with typhoid immune serum. He split off from the germs an active substance which is not ordinary proteid, and which burns with an odor like that of subliming leucin. Madsen, Weingeroff, Bulloch and Hunter, and Kayser are among those who have investigated bacterial hemolysins. Buxton gives a very clear outline of the present theories of immunity, and describes the action of bacteriolysins as directed not against the bacillus as a whole, but against certain albumins in the germ cell. The intermediary body has affinity for these albumin molecules and unites with them; the complement unites with the intermediary body and can then by virtue of its enzymotic nature dissolve or destroy the albumin molecules to such an extent that the bacillus is destroyed. He adds that as it is generally accepted that no antibodies are formed against compounds simpler than albumins, this raises the question whether endotoxins are albuminous or simpler.

EXPERIMENTAL.

MATERIAL. The material used for this work is *Bacillus coli communis* obtained from normal feces, and made more virulent by repeated passage through animals. The large copper tanks in use in this laboratory were filled with ordinary 2 per cent.

agar medium, sterilized, and inoculated with virulent cultures of the colon germ. After seven to fourteen days, the growth was scraped from the agar by means of bent glass rods, a very little water added if necessary, and the germs drawn off into flasks by suction. The cell substance was precipitated by pouring into 95 per cent. alcohol. It was filtered, repeatedly washed with alcohol, extracted with ether in a Soxhlet, dried first between filter papers, and then *in vacuo* over sulfuric acid. When thoroughly dry the germs were pulverized in a porcelain mortar, then in an agate one, and passed through a fine sieve. In this way a light gray or cream-colored powder was obtained which is very nearly pure cell substance without the fat.

MOISTURE AND ASH. The cell substance thus prepared takes up moisture readily and holds it tenaciously, but may be dried to constant weight by heating small amounts in a steam-drying oven for many days at from 85° to 95° C. If the temperature runs down to 60°, it may absorb moisture even in the oven. One sample was heated to 105° during working hours for two or three days, and kept in a desiccator during the intervals. Under this treatment it steadily increased in weight. Drying *in vacuo* over sulfuric acid is on the whole the most satisfactory method, although it requires many days or even weeks.

The dried cell substance burns with a flame, forming volatile and liquid products, giving off odors characteristic of nitrogen compounds, and finally leaving a greenish ash. Two determinations gave the following results:

0.346 gm. of substance gave 0.0296 gm. of ash, or 8.55 per cent.

0.496 gm. of substance gave 0.0431 gm. of ash or 8.68 per cent.

Values reported for other germs vary from 3 per cent. in putrefactive bacteria to 13 per cent. in prodigiosus, or, by using special media, to nearly 30 per cent. in the cholera bacillus; while in the tubercle bacillus the ash has been found to vary from 1.77 per cent. to 5.92 per cent. according to conditions. The ash from the colon bacillus contained sodium, potassium, phosphates, and small amounts of calcium, aluminum, and copper. A slight residue insoluble in acid was probably silica. Sulfates and chlorides were not found. In comparison with the data from other germs, these findings are noteworthy only in the absence of magnesium, and for the presence of copper and

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aluminum. Presumably the copper comes from the tank, and it is very likely that the aluminum comes from the agar.

Phosphorus was the only constituent of the ash quantitatively determined.

The ash was dissolved in nitric acid, the phosphate precipitated with ammonium molybdate, dissolved in ammonia, reprecipitated with magnesia mixture, and weighed as pyrophosphate. The following results were obtained:

Weight of Sample	Weight of Pyrophosphate	Weight of Phosphorus	Per cent. of Phosphorus
0.496 gm.	0.0475 gm.	0.01323 gm.	2.68
0.346 "	0.0380 "	0.01059 "	3.06

The mean of these determinations, 2.87 per cent., agrees quite closely with Levene's finding, 2.67 per cent. of phosphorus in the tubercle bacillus from mannite cultures. Most observers report smaller results, but it should be noted that my samples are free from fat and wax, and therefore the percentages are higher than if calculated for the whole germ.

In view of the number of elementary analyses of different bacteria already reported, and more especially in view of the wide variations in composition caused by the nature of the nutrient medium, the stage of development of the organism, the temperature and other conditions of growth, it did not seem worth while to make determinations of carbon and hydrogen.

PRELIMINARY WORK. Some earlier investigations in this laboratory upon the toxicity of the colon germ showed the desirability of studying the action of dilute acid upon the cell substance. Accordingly samples were treated with 1 per cent sulfuric acid under varying conditions. On filtering, a light brown or straw-colored liquid was obtained. This readily reduced nitric acid, and gave the typical xanthoproteic color on the addition of ammonia. In no case was there more than a slight biuret test, and there was too much sulfate present for a satisfactory Millon test. The α -naphthol test for furfural was positive. Alcohol gave a voluminous precipitate, A, which will be described more fully under another heading. The alcoholic filtrate, B, was neutralized with sodium hydroxid, the sodium

sulfate filtered out together with some organic matter mechanically carried down, and the liquid distilled under diminished pressure at 30°–35° C. The liquid residue, C, left after distillation, turned yellow on heating with potassium hydrate, but gave neither the biuret nor Millon's test. Again, the xanthoproteic and α -naphthol tests were positive, but it failed to reduce either Fehling's or Nylander's solution. It yielded precipitates with ammonium molybdate, phosphomolybdic acid, ammoniacal silver nitrate, and picric acid. A guinea-pig was injected with 5 c. c. of C with no apparent effect.

As there were indications of organic bases, Residue C was tested for xanthin bases in the following manner: on the addition of barium hydrate and barium chlorid it gave a precipitate, D, and a filtrate, E. Precipitate D was warmed with dilute sulfuric acid (1 to 4), and the resulting solution was found to contain carbohydrate, phosphate, and bases. After removing the phosphate, the bases were precipitated by ammoniacal silver nitrate. The silver compound was decomposed by hydrochloric acid. The acid solution gave a gelatinous precipitate with ammonia, soluble in excess of the reagent. Filtrate E was treated with carbon dioxid to remove the barium, and the excess of carbon dioxid removed by boiling. The resulting solution gave tests for furfurol and for bases. It was concentrated to about one-fourth its volume, and precipitated with ammonia and ammoniacal silver nitrate. This precipitate was dissolved in boiling nitric acid, specific gravity 1.1, and allowed to stand forty-eight hours, but no crystals corresponding to the hypoxanthin fraction were found. Accordingly the solution of the silver compounds was concentrated, decomposed with hydrogen sulfid, filtered, the filtrate evaporated, and treated with ammonia. A precipitate was obtained which suggested xanthin. The silver sulfid precipitate was worked up for guanin, which might be carried down with the sulfid, and a small amount of an organic precipitate was obtained. Thus there were indications of the presence of at least two of the xanthin bases, although the amounts were not sufficient for identification. It may be added that larger amounts were obtained as by-products in the work on hexon bases to be described later, and these indications confirmed.

In another set of experiments, Residue C was treated with hot

concentrated barium hydrate until nearly neutral, distilled with magnesia to remove any ammonia formed, more barium hydrate added, and the sulfate filtered out. The filtrate was acidified and xanthin bases precipitated by silver sulfate. The solution, after removing the xanthin bases, was tested for hexon bases according to the method of Kossel and Kutscher, and found to give indications of lysin together with smaller amounts of histidin and arginin.

The residue, left after extracting the moist fresh germ with 1 per cent. sulfuric acid, was thoroughly mixed with 33.33 per cent. acid, and boiled for eight hours in a flask with a reflux condenser. The black extract was filtered, and barium hydrate added until the greater part of the acid was neutralized. After removing the barium sulfate, the filtrate yielded no precipitate with alcohol did not give the biuret test, nor did it reduce Fehling's solution. With nitric acid there was vigorous action, and the addition of ammonia brought out a faint xanthoproteic color. Here again the appropriate tests gave indications of both xanthin and hexon bases.

The residue, after repeated extraction with dilute sulfuric acid (from 1-5 per cent.), was treated with 2-4 per cent. sodium hydrate either upon the water-bath or over a free flame. In every case the substance went into solution readily, leaving only a slight coating on the filter. The slight residue gave no proteid test, contained no nitrogen, but gave test for carbohydrate. In one case it was removed from the filter, and the organic matter approximately determined. The total residue was about 0.4 gram, equivalent to 0.8 per cent. of the cell substance used. The organic matter was only 0.15 gram, equivalent to 0.3 per cent. of the original.

The alkaline extract was neutralized with hydrochloric acid and then poured into 95 per cent. alcohol. A light-colored, flocculent precipitate (G) was obtained. This turned dark on the exposure to air incident to filtration. It was twice dissolved in 0.5 per cent. potassium hydrate and reprecipitated. Each time the fresh precipitate was white or nearly so, but the utmost care in filtering, even in an atmosphere of carbon dioxid, did not prevent its turning dark. The solution in alkali gave the xanthoproteic and furfurol tests, but neither the biuret nor

Millon's test. Copper chlorid gave a precipitate, but picric acid and platinum chlorid did not. The solution was accordingly acidified with picric and acetic acids, copper chlorid added, and the mixture poured into three volumes of alcohol. An aqueous solution of the precipitate thus obtained did not reduce Fehling's solution, but after boiling with hydrochloric acid it reduced both Fehling's and Nylander's solutions, and also gave the furfurol test, thus showing the presence of a carbohydrate. Precipitate G burned readily, puffing up and glowing as does nucleic acid, then fusing and leaving a dark ash. Two determinations of phosphorus gave the following:

Weight of Sample	Weight of Pyrophosphate	Weight of Phosphorus	Percentage of Phosphorus
0.4723 gm.	0.0524 gm.	0.0145 gm.	3.09
0.6469 "	0.0685 "	0.01895 "	2.93

As the amount of carbohydrate was not determined, this simply shows that the nuclein body present contains more than three per cent. of phosphorus. Its behavior, however, points toward nucleic acid.

The work thus far described was merely preliminary, and, like the preliminary tests in ordinary analysis, has shown the action of various solvents sufficing to bring the whole into solution; then to each partial solution a large number of tests (many of those giving negative results not reported in this brief account) were applied to show what classes of compounds might be sought. The amounts used were too small to permit the purification and identification of the compounds obtained, but indications of proteid, carbohydrate, nucleo-compounds, purin and hexon bases have been found. It will be remembered that the fat and wax had been previously removed from the material. From the great number of problems suggested by this preliminary work, two will be reported upon in this paper: a study of the alcoholic precipitate previously designated as A, and the isolation of lysin. While no attempt has been made to isolate the carbohydrate, the abundance of furfurol indicates that pentose, reported in other germs, is present in the colon bacillus as well. No evidence of cellulose was found. In this connection it may

be remarked that in some of the earlier literature the nitrogenous matter was assumed to be simple proteid, and the excess of carbon assumed to give the content of cellulose. Whether the bacterial cell does or does not contain a nucleus, it certainly contains nucleic compounds, and their abundance adds interest to the theory that these cells are mainly nucleus. Indeed, the almost monotonous similarity shown by the extracts prepared under widely varying conditions is very suggestive, especially in conjunction with the lack of differentiation revealed by microscopic study, and with the theory of the bacterial cell as a chemical unit recently advanced by Dr. Vaughan.

A CLEAVAGE PRODUCT. In the preliminary work above described, a cleavage product split off by very dilute acid was deemed worthy of further investigation. Accordingly, the pulverized cell substance after extraction with alcohol was treated with about forty times its weight of 1 per cent. sulfuric acid. Some samples were shaken at ordinary temperatures, while others were heated on the water-bath. The most satisfactory results were obtained by rubbing up the cell substance in a mortar with a little of the acid, then adding acid little by little, with constant stirring, and heating on the water-bath for an hour. On standing, a heavy deposit settled, leaving an opalescent fluid. Various expedients for filtering were tried, a folded hard filter proving the best. With this the first few cubic centimeters come through turbid, but soon the pores of the paper are filled, and the filtrate becomes a clear golden brown. However, the filter gradually becomes clogged, and filtration proceeds more and more slowly. A suction pump is no help, for the pores of the paper are very soon so filled as to stop filtration completely. At best, five days were required to filter two liters.

The filtered acid extract was poured slowly, with constant stirring, into from three to five volumes of 95 per cent. alcohol. The resulting precipitate was white, amorphous, flocculent, and at first very voluminous. This was allowed to settle several hours, filtered, and washed with alcohol until the washings were no longer acid. Prepared thus, the precipitate dissolves in a volume of water equal to the original volume of extract, giving an opalescent solution which filters readily leaving no residue. The longer the precipitate stands in contact with

alcohol, the less readily it redissolves; on the other hand, unless it completely subsides, filtration is slow. The aqueous solution was reprecipitated by pouring into three volumes of absolute alcohol, allowing to settle as before, and filtering as rapidly as possible, because while moist it darkens on exposure to the air. Here again a filter pump was of no assistance, and filtering in an atmosphere of carbon dioxide was not satisfactory. The precipitate was dried between folds of filter paper, and then *in vacuo* over sulfuric acid. It diminished very greatly in bulk on drying, was then hard and horny in texture and gray or brown in color, but when thoroughly pulverized was lighter.

The opalescent solution is acid to litmus, and becomes clear and transparent on the addition of a trace of alkali. It gives a well marked xanthoproteic test, the other color tests for proteid only very slightly. Both phosphotungstic acid and silver nitrate give precipitates, while albumin and ammoniacal silver nitrate do not; no free phosphate ions were found. It gives a good furfural test with α -naphthol, but does not reduce Fehling's solution either before or after boiling with hydrochloric acid.

The cleavage product, like the cell substance, has great affinity for moisture. After standing six months in a bottle with a glass cap, samples absorbed water while being weighed in a watch glass. Small samples (0.5 gm.) dried to constant weight if kept at 98° C. for seven or eight days, or if kept *in vacuo* over sulfuric acid at room temperature for ten days. Larger amounts kept for a few days at 98° C., and then between 105° and 110° C., required still longer, and sometimes showed signs of decomposition before becoming constant.

This substance liquefies on burning, gives off volatile products, and the odor of burning feathers, finally leaving a dark residue. Determinations of the ash are not satisfactory, for particles of organic matter fuse into the residue and can not be consumed. Hammarsten says the nucleins give an acid coke containing metaphosphoric acid which is burned with great difficulty. Very likely this is one trouble here. Further, in trying to burn the organic matter out of the liquid mass, some of the mineral constituents are volatilized. An average of several determinations gives 30.74 per cent. of fixed ash. The amount of inorganic

matter is probably even higher, and includes the metals present in the organism as integral parts of the cell, as well as those taken up from the alkaline culture medium and from the tank. All these would be seized with avidity by the acid used in the extraction, and the sulfates thus formed would appear in the alcoholic precipitate. The ash is not wholly soluble in either nitric or hydrochloric acid. It contains both sulfate and phosphate.

Nitrogen was determined by the Kjeldahl method; sulfur by fusion with potassium hydrate and potassium nitrate, and precipitation as barium sulfate; phosphorus was determined in the ash by the molybdate-magnesia method. A comparison of the results of these determinations with figures obtained from the cell substance is given in the following table.

TABLE III.
COMPARISON OF CELL SUBSTANCE AND CLEAVAGE PRODUCT.

	Ash. Per cent.	Nitrogen. Per cent.	Phosphorus. Per cent.	Sulfur. Per cent.
Cell substance	8.61	10.65*	2.87	None in ash.
Cleavage product	30.74	6.49	5.30	9.22
Do., ash free	—	9.40	—	—

* Air-dried samples not at constant weight were used for this determination.

This substance proves to be toxic, somewhat more toxic, weight for weight, than the whole germ. From a number of animal experiments, the following are reported as typical: One hundred milligrams of the substance were dissolved in 10 c.c. of sterile water to which one drop of sodium hydrate solution was added. Guinea-pigs were injected intra-abdominally at 11:20 A.M., as follows:

No. 1	received	5 c. c.,	corresponding	to	50 mg.	of the substance.
" 2	"	2.5 "	"	"	25 "	"
" 3	"	1 "	"	"	10 "	"
" 4	"	0.5 "	"	"	5 "	"

No. 1 was practically dead at 5 P.M. the same day, although it did not actually draw its last breath until after six. No. 2 died at six P.M., while Nos. 3 and 4 were found dead at 7 o'clock

the next morning. The post-mortem findings were the same as those reported by Vaughan as characteristic of the colon toxin.

Extracts obtained by heating with acid in an autoclave at 140° – 144° C., or by heating the moist fresh germ with acid on a water-bath for two hours, gave precipitates with alcohol which were markedly less toxic. One hundred milligrams of each of these precipitates, dissolved and injected as in the previous experiments, made the animals very sick for a time, but they finally recovered. No chemical differences have been found between the more toxic preparations and the less toxic ones. Behring found tuberculinic acid prepared by extracting the germ with neutral salts or dilute alkali to be five times as toxic as preparations of the acid obtained by boiling the germ with barium hydrate. Levene's experiments showing the instability of tuberculinic acid have already been cited. The variations in toxicity in these preparations from colon and tubercle bacilli may be due to slight differences in chemical composition, or to the instability of the compound and the consequent decomposition of amounts varying with the conditions.

There is abundant evidence of the instability of some of the bacterial toxins. Madsen, for example, found that a 0.1 per cent. solution of tetanolysin in physiological salt solution was weakened by standing an hour at room temperature. Markl had accumulated a quantity of pest toxin for use, and one hot day the temperature of the laboratory was so high as to spoil it. He says that body temperature is unfavorable for this toxin, and suggests that at this temperature toxic substances are formed which in the nascent state unite with the albumin molecule to form an innocuous compound; accordingly in a host this toxin does not accumulate. On the other hand, it has been shown that heating the colon germ with water in an autoclave to 154° C., or in a sealed tube to 184° C., does not destroy its toxicity. Also Sluyts finds cholera toxin, with a chemical nature much like that of the colon toxin, to be quite stable. It can be heated to 120° C. for an hour, or an hour and a half, or exposed to direct sunlight for twenty-four hours, without losing its toxicity.

It seems probable, however, that the toxicity of these colon preparations is due, not to the main body of the substance which responds to the chemical tests, but to the presence of

small amounts of an exceedingly virulent compound mechanically carried down. Wheeler's recent work on the colon toxin serves to confirm this view. Markl believes that attempts to isolate pure toxins fail because of their sensitiveness towards reagents, and also because of the tenacity with which they cling to the albumin molecule, whether acid albumin or abuminate. Brieger says also that if diphtheria toxin is precipitated by any organic or inorganic reagent for precipitating proteid, this simply shows the mechanical carrying down of the toxin by the proteid. Wasserman and Proskauer found toxin carried down by various precipitates, and suggest that the so-called toxalbumin may be a mechanical mixture of toxin and albumose. Roux and Yersin report that a precipitate of calcium phosphate in diphtheria bouillon is toxic, and think the toxicity is due to toxin which clings to the inorganic precipitate. Tichomiroff says that nucleic acid precipitates tetanus and diphtheria toxins.

Aronson, in his work on the diphtheria bacillus, found a great difference between extracts made from fresh material and from material that had been previously exposed to the action of alcohol and ether, and was able to make a distinction between the proteid and the toxin. Using 0.1 per cent. ethylendiamin, he extracted twenty times as much organic matter from fresh germ substance as from material which had previously been extracted with alcohol and ether; equal volumes of the two extracts, however, showed practically the same toxicity. He reasons that the bacteria are so hardened by the alcohol and ether that little proteid goes into solution, while the solubility of the toxin is only slightly affected. If the same ratio holds with these extracts from the colon germ, the animal experiment in which a preparation from the moist germ substance was used, should be compared only with injections of 5 milligrams of the preparations from germs previously extracted with alcohol, usually, but not invariably, killed within twenty-four which hours.

A recent article by Wolff suggests another but less plausible explanation. He regards the intracellular toxins not as an especial class of poisons, but thinks their action is that of any form of foreign albumin when introduced into a host (giftig wie jedes körperfremde Eiweiss). Bacterial proteids, then, are

to be considered denaturized endotoxins, their toxicity coming to light when they are brought into a soluble form that can be absorbed.

The residue, after extraction with acid at ordinary temperatures, was again treated with 1 per cent. acid under the same conditions. The extract was slightly colored, but contained too little organic matter to be worth working up. Again the residue, after extraction with acid on the water-bath, was thoroughly mixed with acid and heated in an autoclave at 138° to 144° C. for an hour. This extract was poured into alcohol, giving a small amount of precipitate, similar to the first, but darker in color and only slightly toxic. The residue from this second extraction was autoclaved for three hours at 140° to 144° C. with 1 per cent. acid, extracting little but humin substance. The residue from the third extraction was autoclaved at 140° to 144° C. for an hour with 5 per cent. acid, again to no avail.

Thus a single extraction at water-bath temperature splits off all that 1 per cent. sulfuric acid can remove from the germ, and it would seem that there is here a definite line of cleavage. The cleavage product insoluble in alcohol as prepared above is confessedly not pure, but even in its impure state permits the drawing of certain conclusions. It is not proteid, as it does not give the proteid tests except the ubiquitous xanthoproteic. The failure of the biuret test shows the absence of protamin. Tests show that it contains sulfate, as would be expected from its preparation; if its sulfur is all combined as sulfate, it contains 27.66 per cent. of SO_4 . On this basis, calculating percentages of nitrogen and phosphorus for compound free from sulfate, we have $\text{N} = 8.98$; $\text{P} = 7.33$. But these figures serve only to give minimal values. The high percentage of phosphorus and the failure of some of the color reactions exclude nucleoproteid and nuclein, but not nucleic acid. Having thus studied a product of cleavage obtained by gentle means, attention was turned to the results of more violent action by which crystalline products could be obtained in a pure state.

HEXON BASES. Since an open chain of six carbon atoms seems to be the favorite foundation for the carbohydrates found in nature, it is a striking coincidence, if nothing more, that

Kossel and others have found the same chain in so many forms of albumin. Moreover in animal metabolism much of the proteid is changed into carbohydrate, and, as Cohnheim suggests, this change can be most easily accomplished by oxidizing preformed chains of six carbon atoms. The preliminary experiments described above showed the presence of basic compounds, presumably hexon, in the cell substance of the colon germ. As one or more of these bases, lysin, arginin, and histidin, have been found in every proteid thus far examined, and in view of Kossel's theory that they are fundamental constituents of proteids, and thus a necessary part of the proteid molecule, and indeed that one of them is the nucleus of that molecule, their presence or absence in the bacterial cell is of considerable theoretical interest.

Lysin, $C_6H_{14}N_2O_2$, α - ϵ -diamido-caproic acid, $NH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$, the base to be especially considered here, was discovered by Drechsel in 1891 among the cleavage products of casein. Ernst Fischer, Siegfried, Kossel, Kutscher, Schulze and Winterstein, Abderhalden, and others have shown its wide distribution in all forms of proteid thus far examined for it, save gliadin, zein, mucedin, gluten fibrin, and certain protamins. Drechsel, Willdenow, and Lawrow prepared the dibenzoyl compound, to which they gave the name lysuric acid; Hedin separated lysin as a silver nitrate double salt; Herzog recommended the formation of a hydantoin by means of phenyl isocyanate; but the separation as picrate by the Kossel and Kutscher method is the one commonly employed. The xanthin bases are first removed by addition of silver nitrate to the acid solution. Histidin is precipitated by silver nitrate and barium hydrate to slight alkalinity; and arginin by excess of silver nitrate and barium hydrate. To the resulting solution phosphotungstic acid is added, precipitating lysin with cholin, betain, and some other compounds. The phosphotungstates are decomposed by barium hydrate, and lysin is precipitated as picrate.

For purposes of comparison, samples of lysin picrate and lysin chlorid were prepared from fibrin and from gelatin. Fibrin was subjected for some weeks to tryptic digestion following the methods of Kühne and Chittenden and of Kutscher. After the

removal of the undecomposed proteid, first by heat, then by successive saturation with ammonium sulfate in acid, in neutral, and in alkaline solution, lysin was separated as outlined above. Also gelatin was boiled for many hours with sulfuric acid, and lysin salts obtained identical with the corresponding preparations from fibrin.

Samples of the cell substance of the colon germ were boiled with acid, and the resulting solution treated by the Kossel and Kutscher method. In the first series of experiments, at every step in the process, indications pointed to the presence of all three of the hexon bases, but at the end, sticky hygroscopic substances were obtained which resisted all attempts to crystallize them. Picric acid gave a waxy or oily mass instead of well defined crystals of lysin picrate. Attempts to make the chlorid either directly or through the picrate, gave a syrupy, amorphous substance, exceedingly hygroscopic, which, after long drying *in vacuo* over sulfuric acid in the cold, was hard and somewhat brittle, but even in a cold room became sticky before it could be pulverized.

In two sets of experiments, in order to trace the decomposition of the proteid through its successive steps, frequent determinations of the amount of nitrogen in the solution were made by the Kjeldahl method. In the first, 173 grams of air-dried cell substance were boiled with nine times its weight of 33.33 per cent. sulfuric acid. This was filtered and the residue repeatedly boiled out with water until the washings gave only a slight precipitate with phosphotungstic acid. The extract and washings contained 18.37 grams of nitrogen, which is 10.62 per cent. of the cell substance used. The residue contained 0.659 gram of nitrogen, 0.38 per cent. of the cell substance. By addition there is then 19.03 grams of nitrogen in the sample used, or 11 per cent. of the germ. Thus the acid has brought into soluble form 96.53 per cent. of the total nitrogen. After precipitating the extract with phosphotungstic acid, there were left in the solution 9.65 grams of nitrogen. The phosphotungstic precipitate then contained 8.715 grams of nitrogen. Following the common assumption that phosphotungstic acid separates the monamido from diamido compounds, these results may be summarized as in Table IV.

TABLE IV.

DISTRIBUTION OF NITROGEN EXTRACT WITH 33.33 PER CENT. ACID.

	Weight in Grams	Percentage of Cell Substance	Percentage of Total N of Cell Substance	Percentage of N of Extract
<i>Cell substance</i>	173.
N in extract	18.37	10.62	96.53	100.
N in residue	0.659	0.38	3.47
Monamido N	9.655	5.581	56.73	52.56
Diamido N	8.715	5.04	45.8	47.44

In another series of experiments the nitrogen was determined at every step. Samples of air-dried cell substance gave 10.65 per cent. of nitrogen. One hundred grams of cell substance, weighed out under similar conditions, were extracted repeatedly with 5 per cent. sulfuric acid. In the extract 9.58 grams of nitrogen were found. Barium hydrate was then added, the sulfate filtered out and boiled with water several times, filtrate and wash water concentrated to the volume of the original extract, about five liters. This contained 8.10 grams of nitrogen; there were then 1.48 grams of humin nitrogen which boiling did not remove from the inorganic precipitate. After acidifying, adding silver nitrate, and filtering, there were left in the filtrate 7.639 grams of nitrogen; thus precipitating the xanthin bases had removed 0.464 gram of nitrogen. After precipitation with silver nitrate and barium hydrate, the solution contained 5.913 grams of nitrogen, 1.726 grams having been removed. After precipitation with phosphotungstic acid, the solution contained 1.704 grams of nitrogen. The silver precipitate for arginin and histidin was rubbed up with water containing sulfuric acid, and decomposed by hydrogen sulfid. To the resulting solution, silver nitrate was added, then barium hydrate to slight alkalinity, to separate the histidin from the arginin. The precipitate was decomposed as before with hydrogen sulfid, the resulting solution containing 0.454 gram of nitrogen. Phosphotungstic acid was added to the solution to separate the histidin from any thymine and uracil that might be present, and the precipitate decomposed by barium hydrate, giving a solution containing 0.358 gram of nitrogen. The solution for arginin contained 0.657 gram of nitrogen, and after the same treatment for separating the py-

When the barium sulfate had completely settled, the supernatant liquid was siphoned off, the precipitate stirred up with boiling water, heated to boiling, settled over night, and again siphoned. This was repeated until the wash water was nearly colorless. The extract and wash water were united, acidified with acetic acid (if there is large excess of barium present, it is well to remove it by carbon dioxid), concentrated on the water-bath, cooled, and filtered to remove any leucin and tyrosin that may crystallize out. The filtrate was diluted to about one and a half liters for each 100 grams of cell substance, made decidedly acid with nitric acid, and 20 per cent. silver nitrate solution added as long as it gave a precipitate. It was left over night to settle, and the silver precipitate of xanthin bases filtered out. To this filtrate excess of silver nitrate and barium hydrate were added to remove arginin and histidin. After their removal, silver and barium were precipitated by hydrochloric and sulfuric acids, these inorganic precipitates boiled out with water several times, the filtrate and wash water united and concentrated. The solution, which should contain some 5 per cent. of acid, was treated with a 50 per cent. solution of phosphotungstic acid as long as it gave an immediate precipitate. The precipitate was rubbed up with 5 per cent. sulfuric acid, carefully washed with the same solution, and filtered with suction. The heavy white precipitate was again rubbed up with water, hot saturated solution of barium hydrate added until the mixture was no longer acid, settled over night, and the supernatant liquid siphoned off. The precipitate consisting of barium phosphate, tungstate, etc., was washed several times with hot barium hydrate solution, decanted, and finally filtered by suction. The filtrate and wash water were united, and barium was removed as carefully as possible, first by running in carbon dioxid, and then by adding ammonium carbonate to the solution. This precipitate, like all the other inorganic ones, was boiled out several times with water, and the washings added to the original filtrate. The resulting liquid was concentrated nearly to dryness on the water-bath, the residue taken up with water, filtered to remove barium carbonate, and again concentrated to a thick syrup.

This alkaline syrup was vigorously stirred with alcohol, and

then with an alcoholic solution of picric acid. Sometimes a crystalline precipitate came down at once, sometimes there was a viscous mass like molasses candy, which became granular or crystalline after long kneading and stirring as Fischer and Weigert suggest. When picric acid would no longer give a precipitate even on standing, the crystals were filtered out by suction, washed with alcohol, and dried on a porous plate. On concentration the alcoholic mother liquor became gummy and viscous, but no more crystals were obtained. The crude picrate was recrystallized from hot water several times. On dissolving there was much sediment which mainly filtered out, but on concentration more appeared upon the sides of the vessel. The loss of substance by the first recrystallization was very large; as it became pure, however, it crystallized like an inorganic salt. All mother liquors were treated with hydrochloric acid to remove picric acid, reprecipitated with phosphotungstic acid, the precipitate worked up as before, and a further crop of crystals obtained. The crystals are slender, yellow, silky, felted needles or prisms. On heating in a melting-point tube, the substance begins to change color at 216° C., and is very dark at 230° C. Heated side by side with lysin picrate from fibrin and from gelatin, they agree within a degree. The authorities all agree that lysin picrate turns black at 230° – 232° C., while Kutscher and Lohmann also say that it begins to change color at 215° C.

To change the picrate into chlorid, 2 grams were dissolved in 30 c.c. of hot water, 5 c.c. of concentrated hydrochloric acid added, cooled, the picric acid filtered out, and washed with water containing hydrochloric acid. The filtrates were shaken out with ether as long as there was any yellow color. The solution should be colorless or nearly so; if it is not, it can be decolorized by treatment with animal charcoal. The solution was evaporated nearly to dryness, first on the water-bath, and finally in a desiccator. When down to a thick syrup, stirring gave crystals. These were recrystallized out of hot water containing hydrochloric acid, giving long colorless prisms, which melt at 192° C., again agreeing with the corresponding salt from gelatin and fibrin. Henze says that lysin chlorid becomes soft at 193° C., and melts at 195° C.; Lawrow says that it has no sharp melting point, but begins to melt at 194° – 195° C.

Henderson collected samples melting from 190° to 200° C. prepared by different individuals from widely different sources. By careful purification he obtained from each sample a product melting at 192°–193° C. Thus it would appear that the apparent discrepancy in melting point is due to impurities. Reactions, crystalline form, properties, and melting (or decomposing) point show that the picrate and chlorid from the germ are identical with lysin picrate and chlorid from gelatin and from fibrin. Thus the presence of one of the hexon bases in the bacterial cell has been established, and another point of resemblance between bacterial and other proteid has been demonstrated.

SUMMARY.

Elementary analyses show that age, conditions of growth, and especially the composition of the nutrient medium cause bacteria of the same strain to differ widely in elementary composition. Proteid, nucleoproteid, nucleic acid, protamin, fat, wax, lecithin, glycogen, and other carbohydrates have all been reported as obtained from the bacterial cell in varying degrees of purity. Cellulose seems to be present in certain species, but by no means in all. Besides the preparations mentioned above, crystalline compounds have been prepared and purified, proving the presence in the cell of xanthin bases, pentose, fatty acids, and perhaps thymine and uracil. Toxins, enzymes, and agglutinins have been split off from the cell; but more progress has been made in determining their physiological action than their chemical nature.

The cell substance of *Bacillus coli communis*, as prepared for this study, yielded about 8.5 per cent. of ash, and 3 per cent. of phosphorus. Although it had been hardened by contact with alcohol, it is completely dissolved by successive treatment with dilute acid and dilute alkali. Heating with 1 per cent. sulfuric acid causes cleavage apparently along definite lines. The extract contains carbohydrate, much phosphorized nitrogenous matter and bases, but neither proteid, protamin, nor nucleoproteid. The decomposition products suggest that the cell is largely made up of nuclein or glyconucleoproteid; but even this dilute acid suffices to break off the carbohydrates, and to split up the proteid. Alcohol precipitates from this extract a hygroscopic,

toxic body, readily acted upon by the air when moist, apparently made up of sulfate, nucleic acid, and colon toxin. The alkaline extract contains carbohydrate and a nucleo-compound. No evidence of cellulose was found.

By digestion with stronger acid, xanthin and hexon bases were obtained. Lysin was isolated as picrate, purified, and transformed into the chlorid, and proved to be identical with lysin picrate and chlorid from other sources.

This investigation was undertaken at the instance of Dr. Victor C. Vaughan, and I wish to express my appreciation of his valuable suggestions and kindly interest. I also make grateful acknowledgment of aid received from the Ford Fellowship fund for carrying on the work.

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THE INFLUENCE OF AUTOLYSIS ON THE PENTOSE CONTENT OF THE PANCREAS.

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The only comprehensive data at present available regarding the content of pentose groups in animal organs and tissues are those published by Grund¹ and Bendix and Ebstein.² The latter investigators noted that human tissues obtained at autopsy contained a relatively smaller percentage of pentoses than the comparable parts removed from animals just slaughtered; and they pointed out that this difference might be attributable not only to the variations in the species studied, but equally well to the fact that the animal materials were investigated in a fresh condition, whereas the human organs were not subjected to analyses earlier than from twenty-four to forty-eight hours post mortem. In pursuit of these ideas, Ebstein³ investigated the changes which the pentose content of organs may experience through the influence of putrefaction, by examining parts of homogeneous tissue pulp after it had stood for varying periods at incubator temperature. The ready decomposition of *free* pentoses under appropriate conditions had already been demonstrated by Bendix⁴ and Salkowski.⁵ Ebstein's experiments plainly indicated the rapid diminution of tissue pentose during putrefaction; the quantities

¹ Grund, *Zeitschr. f. physiol. Chem.* xxxv, p. 111, 1902. The literature on the physiology of the pentoses is reviewed by Neuberg, *Ergebnisse der Physiologie*, iii, Part I, 1904.

² Bendix and Ebstein, *Zeitschr. f. allgem. Physiol.* ii, p. 1, 1902.

³ Ebstein, *Zeitschr. f. physiol. Chem.*, xxxvi, p. 478, 1902.

⁴ Bendix, *Zeitschr. f. diät. u. physikal. Therap.*, iii, Heft 7, 1899.

⁵ Salkowski, *Zeitschr. f. physiol. Chem.*, xxx, p. 478, 1900.

calculated as xylose,¹ in the pancreas for example, decreased from 0.43 per cent. in the fresh organ to 0.18 per cent. after twelve hours, 0.08 per cent. after fifty-five hours, and 0.048 per cent. after one week of decay. Liver showed similar changes during putrefaction.

These observations by no means exclude the participation of non-bacterial, autolytic agencies in the transformation of the tissue pentose-yielding compounds. Neuberg and Milchner² have noted that l-xylose is liberated in autolysis of the pancreas, not however by tryptic digestion of heated pancreas. Autolysis of normal liver failed, in contrast with carcinomatous liver, to yield reducing pentose. No indication is afforded of the actual disintegration of the pentose groups. Beebe and Shaffer³ have determined the pentose content of a few tumors, some of which were doubtless undergoing the autolysis characteristic of these growths. There was, however, no evidence that the degenerated condition of the tissue is accompanied by low pentose figures.

Preliminary to other studies on the physiological rôle of the pentoses I have followed Professor Mendel's suggestion to ascertain what changes a typical pentose-yielding gland like the pancreas experiences in the proportions of this carbohydrate group during antiseptic autolysis. The uniform outcome of these experimental trials indicates that *no pentose is lost during prolonged autolysis in the absence of bacterial agencies. The pentose content of the pancreas is likewise not diminished by peptic digestion of the tissue.* Incidentally the observations of Ebstein on the destructive effects of putrefactive changes have repeatedly been confirmed.

In the experiments upon which the preceding conclusions are based the pentose content of the tissue was determined by the Tollens-Kröber method,⁴ the phloroglucid being dried and weighed in an open Gooch crucible and the pentose calculated

¹ Neuberg has found that the pancreas pentose is l-xylose. Cf. *Ber. d. deutsch. chem. Gesellsch.*, xxxv, p. 1467, 1902.

² Neuberg and Milchner, *Berlin. klin. Wochenschr.*, xli, p. 1081, 1904.

³ Beebe and Shaffer, *Amer. Jour. of Physiol.*, xiv, p. 231, 1905.

⁴ Tollens, *Zeitschr. f. physiol. Chem.*, xxxvi, p. 240, 1902.

as xylose from Kröber's tables.¹ In preparation for the experiments sheep's pancreatic glands were dissected as free from fat as possible, comminuted, and weighed out in equal portions. The control samples were at once put under alcohol and dried; the other portions, suspended in toluol-water, were subjected to the various autolytic changes for different periods of time and the mixtures then dried with addition of alcohol on a water-bath. A few protocols will suffice to indicate the conditions of the experiments and the uniformity of the results. The comparisons are made on the basis of equal weights of the fresh tissues.

INFLUENCE OF AUTOLYSIS ON THE PENTOSE CONTENT OF PANCREATIC TISSUE.

Sample number.	Weight of fresh tissue used. Grams.	Treatment of the tissue.	Weight of phloroglucid obtained. Gram.	Pentose calculated as xylose. Gram.
B	25	Control—dried with alcohol.	0.0705	0.069
B	25	Autolysis—6 weeks in chloroform-water.	0.0692	0.068
D *	50	Control—	0.166	0.156
D *	50	Autolysis in chloroform-water.	0.151	0.143
A	30	Control—	0.109	0.105
A	30	Autolysis—2 weeks in toluol-water.	0.104	0.100
A	30	Autolysis—4 weeks in toluol-water.	0.112	0.107
C †	25	Control—	0.063	0.062
C †	25	Autolysis—9 days in chloroform-water.	0.054	0.054

* The amounts of tissue used in these trials were too large for more accurate quantitative comparisons.

† In these trials the phloroglucid was weighed on dried papers—a method which is less satisfactory than the one described above.

In contrast with these insignificant changes in samples of a tissue difficult to obtain more uniform in composition are typical data furnished by putrefaction experiments. For example :

15 grams of pancreas, analyzed fresh, yielded	0.053 gm. xylose
" " putrefied at room temperature 2 days	0.043 " "
" " " " " 9 "	trace
" " " " " 23 "	none

¹ Kröber, *Ibid.*, xxxvi, Appendix, 1902.

Fresh, unheated, pancreatic tissue was also subjected to digestion with pepsin in dilute hydrochloric acid without appreciable loss of pentose. In these experiments it was necessary to take into account the pentose content of enzyme preparations, to which Friedenthal¹ and Nencki and Sieber² have directed special attention. The commercial pepsin (Parke, Davis and Co.) which was used furnished furfural equivalent to 9.3 per cent. of pentose (calculated as xylose). In one peptic digestion experiment thirty grams of fresh tissue, yielding 0.105 gram of xylose, contained 0.155 gram of pentose after one week's treatment with the enzyme and acid, and a comparable sample gave 0.153 gram of xylose after two weeks' digestion. A more exact balance was instituted in the following experiment: An artificial gastric juice containing 1.1 grams of pepsin per liter of 0.2 per cent. hydrochloric acid was prepared. Five hundred cubic centimeters were allowed to digest thirty grams of fresh pancreatic tissue at 38° C. for a week, an equal portion of the pepsin solution alone being reserved at the same temperature. Both solutions were then neutralized and evaporated to dryness, and the pentose content compared with that of the fresh tissue, with the following results:

The digestion mixture contained	0.141 gm. xylose
The artificial gastric juice alone contained	0.047 " "
Difference	0.094 " "
The fresh gland contained	0.096 " "

¹ Friedenthal, *Arch. f. Physiol.*, p. 181, 1900.

² Nencki and Sieber, *Zeitschr. f. physiol. Chem.*, xxxii, p. 291, 1901; M. Nencki, *Opera omnia*, ii, p. 824.

STUDIES IN THE CHEMISTRY OF THE ION-PROTEID COMPOUNDS.

(Third Communication.¹)

III. ON THE INFLUENCE OF ELECTROLYTES UPON THE TOXICITY OF ALKALOIDS.²

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PLATES II—IV.

I. INTRODUCTORY.

In a recent paper³ I have advanced an hypothesis as to the influence of the ion in the ion-proteid molecule upon the power of such a molecule to combine with acids and bases, and I have advanced evidence, afforded by experiments on the influence of electrolytes upon the staining of tissues by iodine-eosin and by methyl green, in favor of my hypothesis. The experiments alluded to also, in my opinion, afford confirmatory evidence of the actual existence of these compounds. In order to avoid repetition I will only briefly recapitulate the argument which led to this hypothesis. For a somewhat fuller discussion of the question I must refer the reader to the paper to which I have alluded.

The ion-proteid hypothesis, as suggested by Loeb and Pauli,⁴ is that ions, on diffusing into a tissue, enter into combination with some constituents of the tissue, presumably proteids—and on the provisional assumption that these components of

¹The first of these studies appeared in *Pflüger's Arch. f. d. ges. Physiol.*, cx, p. 610, 1905; the second appeared in the last number of this Journal.

²A preliminary report of these experiments appeared in the *University of California Publications*, ii, No. 17, 1905.

³This Journal, i, p. 279, 1906.

⁴J. Loeb, *Amer. Journ. of Physiol.*, iii, p. 327, 1900. W. Pauli, *Ueber Physikalisch-Chemische Methoden und Probleme in der Medizin*, Wien, 1900.

the tissue are proteids the name *ion-proteids* was conferred upon the compounds.

From the intimate dependence of life-phenomena upon the nature of the ions present in the tissue which the numerous recent investigations upon the physiological effects of electrolytes have revealed to us it would appear that if the ion-proteid hypothesis be correct, then the properties of the ion-proteid molecule depend to a considerable extent upon the nature of the ion in combination.

In former papers¹ I have brought forward evidence in favor of the view that the proportions of the different ion-proteids in a tissue depend on the relative *masses* of the ions which are acting on the proteid, and I suggested that the nature of the ion-proteid formed under the influence of an electrolyte might be determined, in general, by the relative velocities of the ions into which the electrolyte dissociates—since the swifter-moving ions would diffuse into the tissue in greater numbers than the slower-moving ions—and the ions present in the tissue in the greater mass might be expected, in general, to take the greater share of the proteid.

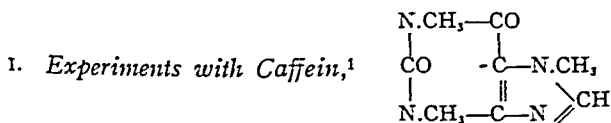
Supposing this hypothesis to be correct then, if the properties of the ion-proteid molecule depend chiefly upon those of the ion in the combination, when an electrolyte is diffusing into a tissue, if the positive ion, whether metal or hydrogen, has a higher velocity than the negative ion, whether an acid radical or hydroxyl, we should expect the resulting ion-proteids to be chiefly *basic* in character in the sense that they should have the power of combining with acids—for the ion in combination is a basic radical and might be expected to confer basic characters upon the ion-proteid molecule—just as one or more methyl groups in the place of hydrogen atoms enhance the basic character of phosphine. Similarly, when the negative ion of the electrolyte has a higher velocity than the positive ion, we should expect to find the resulting ion-proteids in the tissue chiefly acid in character in the sense that they should have the power conferred upon them of combining with bases.

¹ *Trans. Roy. Soc. of South Australia*, xxix, p. 1, 1905. *Pflüger's Arch. f. d. ges. Physiol.*, cx, p. 610, 1905.

The first method of testing the correctness of this hypothesis which suggested itself to me was to investigate the action of alkaloids upon protoplasm which had been subjected to the action of different saline solutions. Since the alkaloids are all basic in character while many of them have also pronounced phenolic or acid characters, their power of combining with a tissue should be profoundly modified by the basic or acid character of the ion-proteids present in the tissue. And, since their toxicity presumably depends upon their power of combining with the tissue, we should expect to find the toxicity of an alkaloid varying according to the electrolyte with which the tissue had been treated.

I had not proceeded far in this investigation when it struck me that it should be possible to reveal the acid or basic characters of a tissue by means of the depth of staining by a color-acid or color-base; as I have said, a first communication of my experimental results in this direction appeared in the last number of this Journal. These results, which, so far as I have gone, are in support of the hypothesis, appeared to me to render the significance of the results of the experiments on alkaloids so much clearer that I decided to publish the two sets of results at the same time, although the investigations on the influence of electrolytes upon the staining of tissues by color-acids and color-bases are as yet incomplete.

II. EXPERIMENTAL.



(a) PREVIOUS INVESTIGATIONS.²

Brunton and Cash³ have investigated the influence of acids and alkalies upon the rigor which caffeine induces in striated

¹ The structural formulæ in this paper are quoted from Pictet, *The Vegetable Alkaloids*, revised and translated by H. C. Biddle, 1904.

² I refer only to previous investigations, of which I have been able to find an account, upon the influence of electrolytes upon the physiological action of the alkaloid under consideration.

³ *Journ. of Physiol.*, ix, p. 112, 1888.

muscle. They found that lactic acid assists while potassium hydroxide inhibits the action of the caffein.

(b) EXPERIMENTS UPON PARAMÆCIUM.

A small, measured amount (about 0.05 c.c.) of culture-fluid containing paramœcia was placed in a paraffin cell of known content (about 1 c.c.), and the cell was then filled with the solution the action of which it was desired to ascertain and the whole immediately covered with a cover-glass. These conditions of volume of culture-medium, volume of solution, etc., were constant throughout the experiments on paramœcium described in this paper, while the temperature was approximately constant, only varying a few degrees. The criterion of death was the cessation of ciliary motion.

In $\frac{N}{50}$ BaCl_2 + $\frac{N}{10000}$ caffein nearly all the paramœcia were dead in 5 minutes—in 10 minutes only one or two individuals were left alive. The average death-time was not exactly ascertained.

In $\frac{N}{33}$ Na_2SO_4 + $\frac{N}{10000}$ caffein by far the majority of the infusoria were still normal in two hours—only one or two individuals out of several hundreds being at all injured.

The toxicity of $\frac{N}{50}$ barium chloride and of $\frac{N}{50}$ sodium sulphate for paramœcia are given on page 529; since barium chloride is so much more toxic than sodium sulphate the above data do not enable us to determine whether or no caffein is more toxic for paramœcia in solutions of barium chloride than in solutions of sodium sulphate.

(c) EXPERIMENTS WITH TUBIFEX.

A small number (20 to 30) of the worms, with as little as possible of the fluid in which they had been living adhering to them, were placed in a finger-bowl containing 55 c.c. of the medium the action of which was being investigated. $\frac{N}{50}$ solutions of salts + $\frac{N}{1000}$ caffein were used. In each case a control experiment was made simultaneously in which the $\frac{N}{50}$ solution of the salt alone was used. The worms had all been obtained from the same locality and were chosen as alike in size and vigor as possible. The criterion of death was cessation of movement on slight shaking.

In $\frac{N}{50}$ MgCl_2 , one or two motionless individuals were first observed in 8 hrs. 10 min., but after 32 hrs. 20 min. slight movements were still observable in the remainder.

In $\frac{N}{50}$ CaCl_2 , slight movements were still observed in 27 hrs. 20 min., but in 32 hrs. 20 min. all were motionless.

In $\frac{N}{50}$ K_2SO_4 , several motionless individuals were observed in 5 hrs. 10 min.; in 8 hrs. 10 min. only slight movements could be detected in one or two; in 10 hrs. 30 min. all were motionless.

In $\frac{N}{12}$ CH_3COONa , slight movements could still be detected in all the worms after 32 hrs. 20 min.; the observations were not continued.

In $\frac{N}{12}$ MgCl_2 + $\frac{M}{1000}$ caffein, two or three motionless individuals were observed in 6 hrs. 50 min.; in 8 hrs. 10 min. only a few were moving, but total cessation of movement was only observed after 32 hrs. 20 min.

In $\frac{N}{12}$ CaCl_2 + $\frac{M}{1000}$ caffein, slight movements were still observed after 27 hrs. 20 min.; by 32 hrs. 20 min. all were motionless.

In $\frac{N}{12}$ K_2SO_4 + $\frac{M}{1000}$ caffein, one individual was motionless in 3 hrs. 5 min.; 40 minutes later, three were motionless; in 8 hrs. 10 min. only one or two were moving; in 10 hrs. 30 min. all were motionless.

In $\frac{N}{12}$ CH_3COONa + $\frac{M}{1000}$ caffein, three were motionless in 5 hrs. 10 min.; several more were motionless at 7 hrs. 10 min., but many were still moving slightly after 32 hrs. 20 min.

Summing up these results, the order of toxicity in the salts alone, as judged by the cessation of movement, was as follows: potassium sulphate, calcium chloride, magnesium chloride, sodium acetate; while in the salts plus caffein the order of toxicity was potassium sulphate, magnesium chloride, calcium chloride, sodium acetate. This appears to me to indicate that magnesium chloride favors the toxic action of caffein to a greater extent than the other salts employed.

(d) EXPERIMENTS WITH GAMMARUS.¹

(2) Ten individuals as alike as possible in size, etc., and from the same locality were carefully freed from adherent fluid by placing them on filter-paper and then placed in one of the following solutions (ten individuals being placed in each solution): $\frac{N}{12}$ CaCl_2 , $\frac{N}{12}$ NaCl , $\frac{N}{12}$ BaCl_2 , $\frac{N}{12}$ NH_4Cl , $\frac{N}{12}$ K_2SO_4 , $\frac{N}{12}$ CH_3COONa , $\frac{N}{12}$ CaCl_2 + $\frac{M}{1000}$ caffein, $\frac{N}{12}$ NaCl + $\frac{M}{1000}$ caffein, $\frac{N}{12}$ BaCl_2 + $\frac{M}{1000}$ caffein, $\frac{N}{12}$ NH_4Cl + $\frac{M}{1000}$ caffein, $\frac{N}{12}$ K_2SO_4 + $\frac{M}{1000}$ caffein, $\frac{N}{12}$ CH_3COOK + $\frac{M}{1000}$ caffein. As in all the experiments on Gammarus described in this paper, the criterion of death was the total cessation of movement upon shaking the finger-bowl containing the solution and the organisms; during the first three or four hours observations were made every half hour. During the following 24 hours observations were made at first every hour and later every two or three hours. Later, observations were made as frequently as possible, the interval between two observations scarcely ever exceeding eight hours, generally being under four hours. In solutions in which the toxicity is very low, as, for example, $\frac{N}{12}$ NaCl , an element of error, besides that due to evaporation, is introduced by the cannibalistic propensities of the organisms. Unless the individual which was eaten had been previously noted as dead, it was omitted in determining the death-time and the average death-time was calculated

¹Gammarus pulex de Geer.

for the remainder. The "toxicity" in the tables following is the reciprocal of the average death-time in minutes.

The following is a table of the results obtained:

TABLE I.

Solution.	Toxicity.	Solution.	Toxicity.
$\frac{N}{52} \text{CaCl}_2$	16×10^{-5}	$\frac{N}{52} \text{CaCl}_2 + \frac{N}{8000} \text{cafein}$	17×10^{-5}
$\frac{N}{52} \text{NaCl}$	0*	$\frac{N}{52} \text{NaCl} + \text{ " }$	0
$\frac{N}{52} \text{BaCl}_2$	80×10^{-5}	$\frac{N}{52} \text{BaCl}_2 + \text{ " }$	80×10^{-5}
$\frac{N}{52} \text{NH}_4\text{Cl}$	71×10^{-5}	$\frac{N}{52} \text{NH}_4\text{Cl} + \text{ " }$	82×10^{-5}
$\frac{N}{52} \text{K}_2\text{SO}_4$	108×10^{-5}	$\frac{N}{52} \text{K}_2\text{SO}_4 + \text{ " }$	154×10^{-5}
$\frac{N}{52} \text{CH}_3\text{COONa}$	12×10^{-5}	$\frac{N}{52} \text{CH}_3\text{COONa} + \text{ " }$	11×10^{-5}

* Less than 1 and greater than zero.

In order to obtain an estimate of the influence exerted by the electrolyte upon the toxicity of the alkaloid itself we must subtract the toxicity of the salt alone from that of the salt + alkaloid—doing this we obtain the following table:

TABLE II.

Salt.	Toxicity of $\frac{N}{8000}$ Caffein + Salt — Toxicity of Salt.
$\frac{N}{52} \text{CaCl}_2$	1×10^{-5}
$\frac{N}{52} \text{NaCl}$	0
$\frac{N}{52} \text{BaCl}_2$	0
$\frac{N}{52} \text{NH}_4\text{Cl}$	11×10^{-5}
$\frac{N}{52} \text{K}_2\text{SO}_4$	46×10^{-5}
$\frac{N}{52} \text{CH}_3\text{COONa}$	-1×10^{-5}

Thus we see that in the case of sodium acetate the addition of $\frac{N}{8000}$ caffeine diminishes the toxicity of the salt itself.

(ii) Ten individuals as alike as possible in condition and size, from the same locality, and carefully freed from adherent fluid by means of filter-paper, were placed in 52 c.c. of each of the following solutions:

- I 50 c.c. $\frac{N}{10} \text{Na}_2\text{HPO}_4 + 2 \text{ c.c. } \text{H}_2\text{O}$
- II 40 c.c. $\frac{N}{10} \text{Na}_2\text{HPO}_4 + 10 \text{ c.c. } \frac{N}{10} \text{NaH}_2\text{PO}_4 + 2 \text{ c.c. } \text{H}_2\text{O}$
- III 30 c.c. $\frac{N}{10} \text{Na}_2\text{HPO}_4 + 20 \text{ c.c. } \frac{N}{10} \text{NaH}_2\text{PO}_4 + 2 \text{ c.c. } \text{H}_2\text{O}$
- IV 20 c.c. $\frac{N}{10} \text{Na}_2\text{HPO}_4 + 30 \text{ c.c. } \frac{N}{10} \text{NaH}_2\text{PO}_4 + 2 \text{ c.c. } \text{H}_2\text{O}$

- V 10 c.c. $\frac{N}{10}$ Na_2HPO_4 + 40 c.c. $\frac{N}{10}$ NaH_2PO_4 + 2 c.c. H_2O
 VI 50 c.c. $\frac{N}{10}$ NaH_2PO_4 + 2 c.c. H_2O
 VII 50 c.c. $\frac{N}{10}$ Na_2HPO_4 + 2 c.c. $\frac{N}{100}$ caffeine
 VIII 40 c.c. $\frac{N}{10}$ Na_2HPO_4 + 10 c.c. $\frac{N}{10}$ NaH_2PO_4 + 2 c.c. $\frac{N}{100}$ caffeine
 IX 30 c.c. $\frac{N}{10}$ Na_2HPO_4 + 20 c.c. $\frac{N}{10}$ NaH_2PO_4 + 2 c.c. $\frac{N}{100}$ caffeine
 X 20 c.c. $\frac{N}{10}$ Na_2HPO_4 + 30 c.c. $\frac{N}{10}$ NaH_2PO_4 + 2 c.c. $\frac{N}{100}$ caffeine
 XI 10 c.c. $\frac{N}{10}$ Na_2HPO_4 + 40 c.c. $\frac{N}{10}$ NaH_2PO_4 + 2 c.c. $\frac{N}{100}$ caffeine
 XII 50 c.c. $\frac{N}{10}$ NaH_2PO_4 + 2 c.c. $\frac{N}{100}$ caffeine¹

The toxicities of the solutions were calculated in the usual way.

The following is a table of the results:

TABLE III.

Solution.	Toxicity.	Solution.	Toxicity.
I	123×10^{-5}	VII	105×10^{-5}
II	139×10^{-5}	VIII	110×10^{-5}
III	112×10^{-5}	IX	122×10^{-5}
IV	85×10^{-5}	X	64×10^{-5}
V	89×10^{-5}	XI	57×10^{-5}
VI	107×10^{-5}	XII	89×10^{-5}

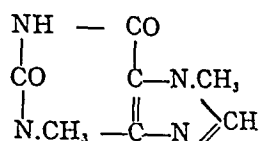
Subtracting the toxicities of the solutions of phosphates alone (I, II, etc., to VI) from those of the phosphates + caffeine so as to ascertain the influence of the phosphates upon the toxicity of the caffeine itself we have:

TABLE IV

Saline Solution.	Toxicity of $\frac{N}{2500}$ Caffein + Saline Solution — Toxicity of Saline Solution
I	-18×10^{-5}
II	-29×10^{-5}
III	10×10^{-5}
IV	-21×10^{-5}
V	-32×10^{-5}
VI	-18×10^{-5}

We see that in all these phosphate solutions except III the addition of $\frac{N}{2500}$ caffeine diminished the toxicity of the solution to a more or less marked degree.

¹ The decinormal solutions of Na_2HPO_4 and NaH_2PO_4 used in these experiments were kindly placed at my disposal by Dr. Cottrell who had made them up himself from Kahlbaum's C. P. salts.

II. *Experiments with Theobromin,*

Theobromin is caffein in which one methyl group has been replaced by a hydrogen atom. Unlike caffein, which possesses only basic characters, theobromin possesses acid properties.

(a) EXPERIMENTS WITH GAMMARUS.

Ten individuals as alike as possible in size, condition, etc., from the same locality and carefully freed from adherent fluid were placed in 52 c.c. of each of the following solutions in finger-bowls: $\frac{N}{52}$ CaCl₂ + $\frac{M}{2000}$ theobromin, $\frac{N}{52}$ NaCl + $\frac{M}{2000}$ theobromin, $\frac{N}{52}$ BaCl₂ + $\frac{M}{2000}$ theobromin, $\frac{N}{52}$ NH₄Cl + $\frac{M}{2000}$ theobromin, $\frac{N}{52}$ K₂SO₄ + $\frac{M}{2000}$ theobromin, $\frac{N}{52}$ CH₃COONa + $\frac{M}{2000}$ theobromin. The controls with salts alone were the same as in the experiments (d i) with caffein. The following is a table of the results obtained.

TABLE V.

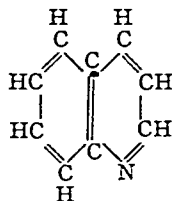
Solution.	Toxicity.	Solution.	Toxicity.
$\frac{N}{52}$ CaCl ₂	16×10^{-5}	$\frac{N}{52}$ CaCl ₂ + $\frac{M}{2000}$ theobromin	19×10^{-5}
$\frac{N}{52}$ NaCl	0	$\frac{N}{52}$ NaCl + " "	0
$\frac{N}{52}$ BaCl ₂	80×10^{-5}	$\frac{N}{52}$ BaCl ₂ + " "	67×10^{-5}
$\frac{N}{52}$ NH ₄ Cl	71×10^{-5}	$\frac{N}{52}$ NH ₄ Cl + " "	73×10^{-5}
$\frac{N}{52}$ K ₂ SO ₄	108×10^{-5}	$\frac{N}{52}$ K ₂ SO ₄ + " "	239×10^{-5}
$\frac{N}{52}$ CH ₃ COONa	12×10^{-5}	$\frac{N}{52}$ CH ₃ COONa + $\frac{M}{2000}$ " "	11×10^{-5}

Subtracting the toxicity of the salt alone from that of the salt + alkaloid we have:

TABLE VI.

Salt.	Toxicity of Salt + $\frac{M}{2000}$ Theobromin — Toxicity of Salt.
$\frac{N}{52}$ CaCl ₂	3×10^{-5}
$\frac{N}{52}$ NaCl	0
$\frac{N}{52}$ BaCl ₂	-13×10^{-5}
$\frac{N}{52}$ NH ₄ Cl	2×10^{-5}
$\frac{N}{52}$ K ₂ SO ₄	131×10^{-5}
$\frac{N}{52}$ CH ₃ COONa	-1×10^{-5}

Thus we see that the addition of $\frac{N}{5000}$ theobromin to $\frac{N}{50}$ barium chloride or to $\frac{N}{50}$ sodium acetate diminished the toxicity of the salts themselves.



III. Experiments with Quinolin,

Quinolin is a tertiary base and does not possess acid characters.

(a) EXPERIMENTS WITH GAMMARUS.

Ten individuals as alike as possible and from the same locality were freed from adherent fluid and placed in 52 c.c. each of the following solutions: $\frac{N}{52}$ CaCl_2 + $\frac{M}{1250}$ quinolin, $\frac{N}{52}$ NaCl + $\frac{M}{1250}$ quinolin, $\frac{N}{52}$ BaCl_2 + $\frac{M}{1250}$ quinolin, $\frac{N}{52}$ NH_4Cl + $\frac{M}{1250}$ quinolin, $\frac{N}{52}$ K_2SO_4 + $\frac{M}{1250}$ quinolin, $\frac{N}{52}$ CH_3COONa + $\frac{M}{1250}$ quinolin. The following is a table of the results obtained—the controls with salts alone being the same as in the experiments (d i) with caffeine.

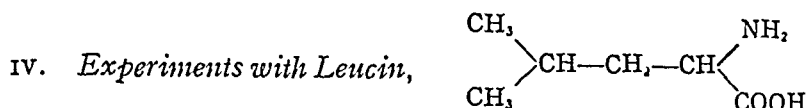
TABLE VII

Solution.	Toxicity.	Solution	Toxicity
$\frac{N}{52}$ CaCl_2	16×10^{-5}	$\frac{N}{52}$ CaCl_2 + $\frac{M}{1250}$ quinolin	29×10^{-5}
$\frac{N}{52}$ NaCl	0	$\frac{N}{52}$ NaCl + " "	13×10^{-5}
$\frac{N}{52}$ BaCl_2	80×10^{-5}	$\frac{N}{52}$ BaCl_2 + " "	113×10^{-5}
$\frac{N}{52}$ NH_4Cl	71×10^{-5}	$\frac{N}{52}$ NH_4Cl + " "	124×10^{-5}
$\frac{N}{52}$ K_2SO_4	108×10^{-5}	$\frac{N}{52}$ K_2SO_4 + " "	321×10^{-5}
$\frac{N}{52}$ CH_3COONa	12×10^{-5}	$\frac{N}{52}$ CH_3COONa + " "	66×10^{-5}

Subtracting the toxicity of the solution of the salt alone from that of the salt + alkaloid, we have:

TABLE VIII

Salt	Toxicity of Salt + $\frac{M}{1250}$ Quinolin — Toxicity of Salt
$\frac{N}{52}$ CaCl_2	13×10^{-5}
$\frac{N}{52}$ NaCl	13×10^{-5}
$\frac{N}{52}$ BaCl_2	33×10^{-5}
$\frac{N}{52}$ NH_4Cl	53×10^{-5}
$\frac{N}{52}$ K_2SO_4	213×10^{-5}
$\frac{N}{52}$ CH_3COONa	54×10^{-5}



Leucin is the α -amino-derivative of isobutylacetic acid and therefore possesses acid as well as basic properties.

(a) EXPERIMENTS WITH GRAMMARUS.

Ten individuals as alike as possible were taken from the same locality and after carefully freeing from adherent fluid were placed in 52 c.c. each of the following solutions: $\frac{N}{52}$ CaCl_2 + $\frac{N}{250}$ leucin, $\frac{N}{52}$ NaCl + $\frac{N}{250}$ leucin, $\frac{N}{52}$ NH_4Cl + $\frac{N}{250}$ leucin, $\frac{N}{52}$ K_2SO_4 + $\frac{N}{250}$ leucin, $\frac{N}{52}$ CH_3COONa + $\frac{N}{250}$ leucin. The controls with salts alone were the same as in experiments (d i) with caffeine. The following is a table of the results obtained.

TABLE IX.

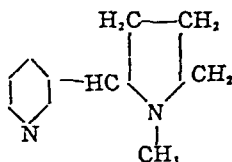
Solution.	Toxicity.	Solution.	Toxicity.
$\frac{N}{52}$ CaCl_2	16×10^{-5}	$\frac{N}{52}$ CaCl_2 + $\frac{N}{250}$ leucin	14×10^{-5}
$\frac{N}{52}$ NaCl	0	$\frac{N}{52}$ NaCl + " "	4.5×10^{-5}
$\frac{N}{52}$ NH_4Cl	71×10^{-5}	$\frac{N}{52}$ NH_4Cl + " "	85×10^{-5}
$\frac{N}{52}$ K_2SO_4	108×10^{-5}	$\frac{N}{52}$ K_2SO_4 + " "	184×10^{-5}
$\frac{N}{52}$ CH_3COONa	12×10^{-5}	$\frac{N}{52}$ CH_3COONa + " "	10×10^{-5}

Subtracting, as usual, the toxicity of the salt alone from that of the salt + alkaloid, we have:

TABLE X.

Salt.	Toxicity of Salt + $\frac{N}{250}$ Leucin - Toxicity of Salt.
$\frac{N}{52}$ CaCl_2	-2×10^{-5}
$\frac{N}{52}$ NaCl	4.5×10^{-5}
$\frac{N}{52}$ NH_4Cl	14×10^{-5}
$\frac{N}{52}$ K_2SO_4	76×10^{-5}
$\frac{N}{52}$ CH_3COONa	-2×10^{-5}

Thus we see that the addition of $\frac{N}{250}$ leucin to $\frac{N}{52}$ calcium chloride or to $\frac{N}{52}$ sodium acetate diminished the toxicity of these salts.

v. *Experiments with Nicotin,*

Nicotin is a diacid base and forms salts with one or with two equivalents of acid.

(a) PREVIOUS INVESTIGATIONS.

Pickering¹ has found that the depressant action of nicotin upon the beat of the heart in the chicken embryo is antagonized by potassium chloride, and he states that the same antagonism can be demonstrated on the frog's heart.

(b) EXPERIMENTS WITH PARAMÆCIUM.

In $\frac{N}{50}$ CaCl_2 + $\frac{M}{10000}$ nicotin, several dead and cytolysed individuals were noted in 13 minutes; in 20 minutes most of the infusoria were dead; in 30 minutes one or two only were alive.

In $\frac{N}{50}$ Na_2SO_4 + $\frac{M}{10000}$ nicotin, one or two dead and swollen individuals were noted at the end of 20 minutes; in 26 minutes about 50 per cent. were dead; in 40 minutes only one or two were left alive.

Since the toxicities of $\frac{N}{50}$ calcium chloride and of $\frac{N}{50}$ sodium sulphate alone are too slight to appreciably affect the relative toxicities of the above two solutions (see p. 529) we must conclude that in $\frac{N}{50}$ calcium chloride nicotin is slightly more toxic for paramœcium than it is in $\frac{N}{50}$ sodium sulphate.

(c) EXPERIMENTS WITH TUBIFEX.

(i) From 20 to 30 worms of approximately the same size and condition and from the same locality were placed in finger-bowls containing 55 c.c. each of the following solutions: $\frac{N}{50}$ MgCl_2 , $\frac{N}{50}$ CaCl_2 , $\frac{N}{50}$ CH_3COONa , $\frac{N}{50}$ Na_2SO_4 , $\frac{N}{50}$ MgCl_2 + $\frac{M}{10000}$ nicotin², $\frac{N}{50}$ CaCl_2 + $\frac{M}{10000}$ nicotin, $\frac{N}{50}$ CH_3COONa + $\frac{M}{10000}$ nicotin, $\frac{N}{50}$ Na_2SO_4 + $\frac{M}{10000}$ nicotin.

In $\frac{N}{50}$ MgCl_2 , movements continued to be active and vigorous up to 3 hrs. 10 min.; after 5 hrs. 30 min. slight movements were still to be noted in all the individuals and observations were not continued.

In $\frac{N}{50}$ CaCl_2 , a diminution in the activity of the movements could be plainly observed by 3 hrs. 10 min.; but at 5 hrs. 30 min. the worms appeared to be in about the same condition as those in MgCl_2 .

¹ *Journ. of Physiol.*, xiv, p. 383.

² Through an oversight the concentration of the nicotin was stated to be $\frac{N}{1000}$ in my preliminary communication to which I have referred. I take this opportunity of pointing out that it should have been $\frac{M}{1000}$ as above.

In $\frac{N}{55}$ CH_3COONa , one or two motionless individuals were observed after 2 hrs. 10 min. and the movements of the rest were sluggish. In 5 hrs. 30 min., very slight movements could still be detected in a few.

In $\frac{N}{55}$ Na_2SO_4 , the movements had nearly ceased by 2 hrs. 10 min., and several were quite motionless; in 3 hrs. 10 min. all were motionless.

In $\frac{N}{55}$ MgCl_2 + $\frac{N}{1100}$ nicotin, movements were hardly perceptible in 10 minutes, while in 25 minutes nearly all the worms were motionless. In 1 hr. 10 min. all were motionless save one, and in 1 hr. 30 min. all movements had ceased.

In $\frac{N}{55}$ CaCl_2 + $\frac{N}{1100}$ nicotin, the movements were very slight and some were motionless in 25 minutes; in 35 min. the worms were nearly all motionless; in 1 hr. 10 min. all were motionless except one; in 1 hr. 30 min. movements had ceased in all the worms.

In $\frac{N}{55}$ CH_3COONa + $\frac{N}{1100}$ nicotin, the movements were slight in 25 min., and in 35 min. several were motionless; in 1 hr. 10 min. about 50 per cent. were motionless, and the rest only moving slightly; in 2 hrs. 10 min. all were motionless.

In $\frac{N}{55}$ Na_2SO_4 + $\frac{N}{1100}$ nicotin, the movements were sluggish but evident in 35 min.; in 1 hr. 10 min. the movements were slight; in 3 hrs. 10 min. all the worms were motionless.

The order of toxicity of the salts alone was therefore : sodium sulphate, sodium acetate, calcium chloride, magnesium chloride; while in the salts plus nicotin the order of toxicity was : magnesium chloride, calcium chloride, sodium acetate, sodium sulphate. This appears to indicate that magnesium chloride and calcium chloride favor the toxic action of nicotin to a much greater extent than sodium acetate and sodium sulphate. Certainly the much greater toxicity of the solutions of the former salts plus nicotin than of the solutions of the latter salts plus nicotin cannot be referred simply to the added toxic action of the salt and of the alkaloid, since the alkaloid is most toxic when acting in the least toxic salt.

(ii) As in the previous experiment 20 to 30 of the worms were placed in finger-bowls containing 55 c.c. each of the following solutions: $\frac{N}{55}$ MgSO_4 , $\frac{N}{55}$ NH_4Cl , $\frac{N}{55}$ KNO_3 , $\frac{N}{55}$ CH_3COOK , $\frac{N}{55}$ MgSO_4 + $\frac{N}{1100}$ nicotin, $\frac{N}{55}$ NH_4Cl + $\frac{N}{1100}$ nicotin, $\frac{N}{55}$ KNO_3 + $\frac{N}{1100}$ nicotin, $\frac{N}{55}$ CH_3COOK + $\frac{N}{1100}$ nicotin.

In $\frac{N}{55}$ MgSO_4 slight movements were still observable at the end of 4 hrs. 30 min.

In $\frac{N}{55}$ NH_4Cl , the movements at the end of 4 hrs. 30 min. were very slight.

In $\frac{N}{55}$ KNO_3 , the condition of the worms at the end of 4 hrs. 30 min. appeared to be about the same as in MgSO_4 .

In $\frac{N}{55}$ CH_3COOK the worms were all motionless in 4 hrs. 30 min.

In $\frac{N}{12}$ $MgSO_4$ + $\frac{M}{1160}$ nicotin, the movements were slight in 10 min.; in 40 min. they were very slight, and in 4 hrs. 30 min. had ceased.

In $\frac{N}{12}$ NH_4Cl + $\frac{M}{1160}$ nicotin, the movements were very slight at the end of the first 10 min. and in 40 min. were almost imperceptible; in 50 min. all the worms were motionless.

In $\frac{N}{12}$ KNO_3 + $\frac{M}{1160}$ nicotin, the movements were also very slight in 10 min., almost imperceptible in 40 min., and ceased in 50 min.

In $\frac{N}{12}$ CH_3COOK + $\frac{M}{1160}$ nicotin, the movements were very slight in 40 min., and in 4 hrs. 30 min. only one individual was moving.

The order of the toxicity of the salts alone was therefore: potassium acetate, ammonium chloride, potassium nitrate, magnesium sulphate,—the toxicity of the two last being, as far as was observed, about equal. The order of the toxicity of the salts plus nicotin was ammonium chloride, potassium nitrate, magnesium sulphate, potassium acetate, the toxicity of the two first being about equal. We cannot conclude from this that ammonium chloride is more favorable to the toxic action of nicotin than magnesium sulphate, for magnesium sulphate itself is less toxic than ammonium chloride: but we see that potassium acetate tends to inhibit the toxic action of nicotin, for, while it is the most toxic salt, when acting together with nicotin it is the least toxic.

(iii) As in the previous experiments, 20 to 30 of the worms were placed in finger-bowls containing 55 c.c. each of the following solutions: $\frac{N}{12}$ $MnCl_2$, $\frac{N}{12}$ $BaCl_2$, $\frac{N}{12}$ $NaCl$, $\frac{N}{12}$ K_2SO_4 , $\frac{N}{12}$ $MnCl_2$ + $\frac{M}{1160}$ nicotin, $\frac{N}{12}$ $BaCl_2$ + $\frac{M}{1160}$ nicotin, $\frac{N}{12}$ $NaCl$ + $\frac{M}{1160}$ nicotin, $\frac{N}{12}$ K_2SO_4 + $\frac{M}{1160}$ nicotin.

In $\frac{N}{12}$ $MnCl_2$, the movements continued to be normal and active for 2 hrs. 15 min. In 7 hrs. all the worms were motionless.

In $\frac{N}{12}$ $BaCl_2$, the movements were sluggish in 10 min.; in 25 min. they were very slight, and in 45 min., they had ceased and the worms were considerably disintegrated.

In $\frac{N}{12}$ $NaCl$, slight movements could be detected after 7 hrs.

In $\frac{N}{12}$ K_2SO_4 , the movements continued to be normal in character for about 1 hr. In 1 hr. 30 min. several were motionless and in 2 hrs. 15 min. the movements of the remainder were slight. In 7 hrs. all were motionless.

In $\frac{N}{12}$ $MnCl_2$ + $\frac{M}{1160}$ nicotin, the movements were very slight in 10 min.; in 25 min. nearly all the worms were motionless and the movements of the remainder were barely perceptible; in 45 min. all were motionless.

In $\frac{N}{12}$ $BaCl_2$ + $\frac{M}{1160}$ nicotin, the movements were slight in 10 min.; in 25 min. they were very slight and many were motionless; in 45 min. all were motionless and many were considerably disintegrated.

In $\frac{N}{12}$ $NaCl$ + $\frac{M}{1160}$ nicotin, the movements were slight in 10 min., and

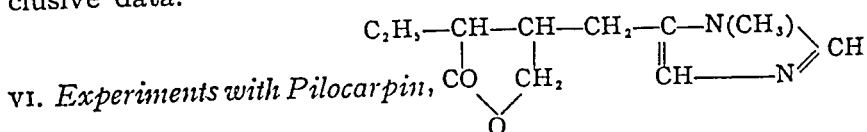
most of the worms were motionless in 25 min.; in 45 min. there were very faint movements on the part of one or two; in 1 hr. only one individual was moving, and in 2 hrs. 15 min. all were motionless.

In $\frac{N}{25} K_2SO_4 + \frac{M}{1000}$ nicotin, the movements were slight in 10 min., and in 25 min. most of the worms were motionless; in 45 min. there were barely perceptible movements on the part of one or two; in 1 hr. one individual was moving and in 1 hr. 30 min. all were motionless.

The order of toxicity of the salts alone was therefore: barium chloride, potassium sulphate, manganous chloride, sodium chloride; while in the salts plus nicotin, the order was: manganous chloride, barium chloride, potassium sulphate, sodium chloride.

While we cannot conclude from this that sodium chloride inhibits the toxic action of the nicotin to a greater extent than the other salts, for sodium chloride is itself the least toxic of the salts under consideration, it is very evident that manganous chloride powerfully aids the toxic action of the nicotin, and that potassium sulphate is unfavorable to the action. Regarding barium chloride we cannot decide from this experiment since its own toxicity is so high.

Summing up the results of these experiments, we conclude that whereas manganous chloride, magnesium chloride, and calcium chloride markedly assist the toxic action of nicotin upon *Tubifex* in the order in which they are written, potassium acetate and potassium sulphate tend to inhibit its action or at any rate to aid it to a very much less degree. Regarding the influence of potassium nitrate, ammonium chloride, sodium acetate, sodium sulphate, sodium chloride, barium chloride, and magnesium sulphate these experiments afford us no conclusive data.



The structural formula of pilocarpin is not absolutely certain, but the above is regarded as the most probable¹; it is a monacid base, but is soluble in alkalis with the formation of salts of

¹ Pictet, *loc. cit.*, from which, unless otherwise stated, the other statements in this paper concerning the chemical properties of the alkaloids are taken.

pilocarpinic acid, the pilocarpin being thrown out of solution again by acids.¹

(a) PREVIOUS INVESTIGATIONS.

Pickering² has found that although pilocarpin produces an atonic standstill in the embryonic heart apparently the same as that produced by nicotin and by veratrin, yet this action of pilocarpin cannot be antagonized by potassium chloride. MacCallum³ has shown that the action of pilocarpin in causing intestinal peristalsis in *Sida crystallina* cannot be antagonized by calcium chloride.

(b) EXPERIMENTS WITH TUBIFEX.

As in the experiments with Tubifex previously described (p. 510), from 20 to 30 of the worms of approximately the same size and condition and from the same locality were freed from adherent fluid and placed in finger-bowls containing 55 c.c. each of the following solutions: $\frac{N}{1150}$ MgCl₂ + $\frac{M}{1150}$ pilocarpin, $\frac{N}{1150}$ CaCl₂ + $\frac{M}{1150}$ pilocarpin, $\frac{N}{1150}$ BaCl₂ + $\frac{M}{1150}$ pilocarpin, $\frac{N}{1150}$ Na₂SO₄ + $\frac{M}{1150}$ pilocarpin, $\frac{N}{1150}$ CH₃COONa + $\frac{M}{1150}$ pilocarpin, $\frac{N}{1150}$ K₂SO₄ + $\frac{M}{1150}$ pilocarpin.

Control experiments with salts alone were not made in this case as the previous control experiments (see p. 517) agree sufficiently well among themselves to indicate in what order the salts alone are toxic for Tubifex.

In $\frac{N}{1150}$ MgCl₂ + $\frac{M}{1150}$ pilocarpin, the movements continued to be active and normal up to 3 hours, at which time a diminution of activity was observed. In 3 hrs. 45 min., the movements were very slight, but they continued for a considerable time in some individuals; all the worms were motionless after 7 hrs.

In $\frac{N}{1150}$ CaCl₂ + $\frac{M}{1150}$ pilocarpin, a diminution in the activity of the movements was observed after 3 hrs. 45 min.; but slight movements continued for some time. In 8 hrs., some were still moving slightly; in 9 hrs., all were motionless.

In $\frac{N}{1150}$ BaCl₂ + $\frac{M}{1150}$ pilocarpin, the movements were sluggish in 10 min.; in 50 min., all were motionless.

In $\frac{N}{1150}$ Na₂SO₄ + $\frac{M}{1150}$ pilocarpin, a diminution in activity was observed after 3 hrs. 45 min.; slight movements continued among the majority until after 5 hrs.; in 6 hrs., some still showed slight movements; in 7 hrs., all were motionless.

In $\frac{N}{1150}$ CH₃COONa + $\frac{M}{1150}$ pilocarpin, the movements remained active and normal for 3 hrs.; in 3 hrs. 45 min., the movements were sluggish but evident, and these movements continued for over 30 hrs. In 45 hrs., all were motionless.

In $\frac{N}{1150}$ K₂SO₄ + $\frac{M}{1150}$ pilocarpin, the movements were normal during the

¹ Beilstein, *Handbuch der organischen Chemie*, iii, p. 924.

² *Journ. of Physiol.*, xiv, p. 383.

³ *University of California Publications, Physiology*, ii, p. 65, 1905.

first hour. At the end of 2 hrs., they were very sluggish. In 3 hrs. 45 min., very slight movements in one or two individuals; in 5 hrs., all were motionless.

Hence the order of toxicity of the salts plus pilocarpin was: barium chloride (most toxic), potassium sulphate, magnesium chloride, sodium sulphate, calcium chloride, sodium acetate; while in the salts alone, referring to the experiments (c) with nicotin and caffein, the order of toxicity for *Tubifex* is: barium chloride, potassium sulphate, sodium acetate, calcium chloride, magnesium chloride; appearing to indicate that magnesium chloride favors the toxic action of pilocarpin to a greater extent than the other salts with the possible exceptions of barium chloride and potassium sulphate. Regarding sodium acetate the controls in the previous experiments show that its toxicity relative to that of magnesium chloride and calcium chloride is not certain, so that we cannot conclude definitely from these results that it tends to antagonize pilocarpin for *Tubifex*.

(c) EXPERIMENTS WITH GAMMARUS.

(1) Five individuals as uniform in size, condition, etc., as possible from the same locality and carefully freed from adherent fluid were placed in 52 c.c. of each of the following solutions, in finger-bowls: $\frac{N}{52}$ $MgSO_4$, $\frac{N}{52}$ $CaCl_2$, $\frac{N}{52}$ $MgCl_2$, $\frac{N}{52}$ $NaCl$, $\frac{N}{52}$ Na_2SO_4 , $\frac{N}{52}$ NH_4Cl , $\frac{N}{52}$ $BaCl_2$, $\frac{N}{52}$ CH_3COONa , $\frac{N}{52}$ K_2SO_4 , $\frac{N}{52}$ CH_3COOK , and in each of these + $\frac{N}{3600}$ pilocarpin. As in the experiments with *Gammarus* previously described, the death-time of each individual was determined and the average duration of life in each solution was thus calculated. The toxicity of the solution was estimated as the reciprocal of the average duration of life of the five individuals. The following is a table of the results obtained:

TABLE XI.

Solution	Toxicity	Solution	Toxicity
$\frac{N}{52}$ $CaCl_2$	33×10^{-5}	$\frac{N}{52}$ $CaCl_2$ + $\frac{N}{3600}$ pilocarpin	113×10^{-5}
$\frac{N}{52}$ $MgCl_2$	0×10^{-5}	$\frac{N}{52}$ $MgCl_2$ + " "	16×10^{-5}
$\frac{N}{52}$ $MgSO_4$	29×10^{-5}	$\frac{N}{52}$ $MgSO_4$ + " "	47×10^{-5}
$\frac{N}{52}$ Na_2SO_4	68×10^{-5}	$\frac{N}{52}$ Na_2SO_4 + " "	55×10^{-5}
$\frac{N}{52}$ $NaCl$	5×10^{-5}	$\frac{N}{52}$ $NaCl$ + " "	1×10^{-5}
$\frac{N}{52}$ $BaCl_2$	56×10^{-5}	$\frac{N}{52}$ $BaCl_2$ + " "	164×10^{-5}
$\frac{N}{52}$ NH_4Cl	106×10^{-5}	$\frac{N}{52}$ NH_4Cl + " "	120×10^{-5}
$\frac{N}{52}$ K_2SO_4	372×10^{-5}	$\frac{N}{52}$ K_2SO_4 + " "	290×10^{-5}
$\frac{N}{52}$ CH_3COONa	14×10^{-5}	$\frac{N}{52}$ CH_3COONa + " "	24×10^{-5}
$\frac{N}{52}$ CH_3COOK	518×10^{-5}	$\frac{N}{52}$ CH_3COOK + " "	350×10^{-5}

Subtracting the toxicity of the solution of the salt alone from that of the salt + pilocarpin, so as to ascertain the manner in which the salt influenced the toxicity of the alkaloid itself, we have:

TABLE XII.

Salt	Toxicity of $\frac{N}{2600}$ Pilocarpin + Salt - Toxicity of Salt.
$\frac{N}{12}$ CaCl ₂	80×10^{-5}
" MgCl ₂	16×10^{-5}
" MgSO ₄	18×10^{-5}
" Na ₂ SO ₄	-13×10^{-5}
" NaCl	-4×10^{-5}
" BaCl ₂	108×10^{-5}
" NH ₄ Cl	14×10^{-5}
" K ₂ SO ₄	-21×10^{-5}
" CH ₃ COONa	10×10^{-5}
" CH ₃ COOK	-168×10^{-5}

Thus we see that in some salts, namely, sodium sulphate, sodium chloride, potassium sulphate, and potassium acetate, the addition of $\frac{N}{2600}$ pilocarpin actually diminished the toxicity of the salt itself.

(ii) These experiments were repeated with a different batch of *Gammarus* from another locality. The details of the experiment were precisely the same as those of the last, only ten individuals were placed in each solution instead of five, so that the duration of life which was determined in these experiments was the average duration of life of ten individuals. The following is a table of the results.

TABLE XIII.

Solution.	Toxicity.	Solution.	Toxicity.
$\frac{N}{12}$ CaCl ₂	41×10^{-5}	$\frac{N}{12}$ CaCl ₂ + $\frac{N}{2600}$ pilocarpin	47×10^{-5}
$\frac{N}{12}$ MgSO ₄	29×10^{-5}	$\frac{N}{12}$ MgSO ₄ + " "	64×10^{-5}
$\frac{N}{12}$ Na ₂ SO ₄	61×10^{-5}	$\frac{N}{12}$ Na ₂ SO ₄ + " "	74×10^{-5}
$\frac{N}{12}$ BaCl ₂	87×10^{-5}	$\frac{N}{12}$ BaCl ₂ + " "	100×10^{-5}
$\frac{N}{12}$ NH ₄ Cl	131×10^{-5}	$\frac{N}{12}$ NH ₄ Cl + " "	87×10^{-5}
$\frac{N}{12}$ K ₂ SO ₄	374×10^{-5}	$\frac{N}{12}$ K ₂ SO ₄ + " "	487×10^{-5}
$\frac{N}{12}$ CH ₃ COOK	343×10^{-5}	$\frac{N}{12}$ CH ₃ COOK + " "	393×10^{-5}

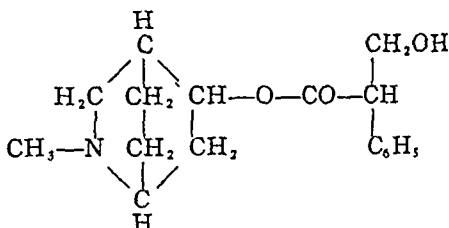
Subtracting, as before, the toxicity of the solution of the salt alone from that of the salt + pilocarpin, we have:

TABLE XIV.

Salt.	Toxicity of Pilocarpin + Salt — Toxicity of Salt.
$\frac{N}{57} \text{CaCl}_2$	47×10^{-5}
" MgSO_4	35×10^{-5}
" Na_2SO_4	13×10^{-5}
" BaCl_2	13×10^{-5}
" NH_4Cl	-44×10^{-5}
" K_2SO_4	113×10^{-5}
" CH_3COOK	50×10^{-5}

Here the addition of $\frac{N}{5700}$ pilocarpin only diminished the toxicity of the salt itself in one case, namely ammonium chloride.

VII. Experiments with Atropin,



Atropin is a tertiary base, but the presence of the CH_2OH group confers upon it the power of combining with bases, the H atom in the hydroxyl being replaceable by metals.

(a) PREVIOUS INVESTIGATIONS.

Zoethout¹ has shown that NaOH diminishes the toxicity of atropin for paramoecia.

(b) EXPERIMENTS WITH TUBIFEX.

As in the experiments with Tubifex previously described, from 20 to 30 of the worms of approximately the same size and condition, and from the same locality and freed from adherent fluid by means of filter paper, were placed in finger-bowls containing 55 c. c. each of the following solutions: $\frac{N}{15} \text{MgCl}_2 + \frac{M}{1100}$ atropin, $\frac{N}{57} \text{CaCl}_2 + \frac{M}{1100}$ atropin, $\frac{N}{57} \text{Na}_2\text{SO}_4 + \frac{M}{1100}$ atropin, $\frac{N}{57} \text{CH}_3\text{COONa} + \frac{M}{1100}$ atropin. No control experiments with salts alone were made in this case.

¹ *Amer. Journ. of Physiol.*, ii, p. 220, 1899.

In $\frac{N}{11}$ $MgCl_2$ + $\frac{M}{1100}$ atropin, the movements continued normal and active for about 4 hrs. In 5 hrs., the movements were slight and in 6 hrs., all were motionless.

In $\frac{N}{11}$ $CaCl_2$ + $\frac{M}{1100}$ atropin, the movements were very slight in 7 hrs.; in 9 hrs., only one or two showed movements; in $\frac{1}{4}$ 1 hrs. 30 min., the movements were barely perceptible; in 20 hrs. 30 min., only one was moving; in 24 hrs., all were dead.

In $\frac{N}{11}$ Na_2SO_4 + $\frac{M}{1100}$ atropin, the movements were active and normal during the first 2 hrs.; in 3 hrs., the movements were sluggish; in 5 hrs., only a few were moving; in 8 hrs., only one was moving, and in 9 hrs. all were motionless.

In $\frac{N}{11}$ CH_3COONa + $\frac{M}{1100}$ atropin, the movements were slight in 20 hrs. 30 min., cessation of movement occurred in between 57 and 70 hrs.

Hence the order of toxicity of these salts plus atropin for Tubifex was: magnesium chloride, sodium sulphate, calcium chloride, sodium acetate; while the order of toxicity of the salts alone, referring to the experiments (c) with nicotin, is sodium sulphate, sodium acetate, calcium chloride, magnesium chloride. Hence we may conclude that magnesium chloride markedly favors the toxic action of atropin upon Tubifex.

VIII. *Experiments with Physostigmin (Eserin), (C₁₅H₂₁N₃O₂)*

The structural formula of physostigmin is not known. It is a monacid base and contains a hydroxyl and two N-methyls.

(a) PREVIOUS INVESTIGATIONS.

Mathews and Brown¹ have found that the action of physostigmin in producing contractions of the intestine and tremors of the voluntary muscles in the dog is counteracted and inhibited by calcium chloride.

(b) EXPERIMENTS WITH PARAMECIUM.

As in the experiments (b) with caffein, a small measured amount of culture fluid containing paramoecia was placed in a paraffin cell of measured content and the cell was then filled up with the solution, the action of which was to be determined, and the whole immediately covered with a cover-glass so that no air-bubbles remained underneath. The criterion of death was cessation of ciliary motion.

In $\frac{N}{11}$ $BaCl_2$ + $\frac{M}{10000}$ physostigmin salicylate, many were dead in 3 min.; in 5 min. most, and in 15 min. all, were dead.

In $\frac{N}{11}$ Na_2SO_4 + $\frac{M}{10000}$ physostigmin salicylate, one or two died in 6 min.; in 10 min., nearly 50 per cent. were dead; in 20 min., many were

¹ *Amer. Journ. of Physiol.*, xii, p. 173, 1904.

still alive, and even after 1 hr. 40 min., a few were alive and apparently normal.

Owing to the fact (see p. 529) that barium chloride itself is much more toxic for paramoecia than sodium sulphate, we cannot definitely conclude from the above experiments what influence the salts used had on the toxicity of physostigmin salicylate for the paramoecia.

(c) EXPERIMENTS WITH GAMMARUS.

(2) As in the experiments (c, 1) with pilocarpin, five individuals as uniform in size, condition, etc., as possible, from the same locality, and freed from adherent fluid, were placed in 52 c. c. of each of the following solutions in finger-bowls: $\frac{N}{52}$ $MgSO_4$ + $\frac{M}{2000}$ physostigmin sulphate, $\frac{N}{52}$ $BaCl_2$ + $\frac{M}{2000}$ physostigmin sulphate, $\frac{N}{52}$ K_2SO_4 + $\frac{M}{2000}$ physostigmin sulphate, $\frac{N}{52}$ CH_3COONa + $\frac{M}{2000}$ physostigmin sulphate. The controls in the salts alone were the same as those in the experiments on pilocarpin to which I have referred. The toxicities of the solutions were calculated as usual. The following is a table of the results obtained:

TABLE XV.

Solution.	Toxicity.	Solution.	Toxicity.
$\frac{N}{52}$ $MgSO_4$	29×10^{-5}	$\frac{N}{52}$ $MgSO_4$ + $\frac{M}{2000}$ physos. sulph	62×10^{-5}
$\frac{N}{52}$ $BaCl_2$	56×10^{-5}	$\frac{N}{52}$ $BaCl_2$ + " " "	145×10^{-5}
$\frac{N}{52}$ K_2SO_4	372×10^{-5}	$\frac{N}{52}$ K_2SO_4 + " " "	472×10^{-5}
$\frac{N}{52}$ CH_3COONa	14×10^{-5}	$\frac{N}{52}$ CH_3COONa + " " "	78×10^{-5}

Subtracting the toxicity of the solution of the salt alone from that of the salt + physostigmin sulphate, we have:

TABLE XVI.

Salt.	Toxicity of $\frac{M}{2000}$ Physostigmin Sulphate + Salt - Toxicity of Salt.
$\frac{N}{52}$ $MgSO_4$	33×10^{-5}
" $BaCl_2$	89×10^{-5}
" K_2SO_4	100×10^{-5}
" CH_3COONa	64×10^{-5}

(ii) The experiment was repeated with five more individuals from the same batch as before in each of the following solutions: $\frac{N}{52}$ $MgSO_4$ + $\frac{M}{2000}$ physostigmin sulphate, $\frac{N}{52}$ $BaCl_2$ + $\frac{M}{2000}$ physostigmin sulphate, $\frac{N}{52}$ CH_3COONa + $\frac{M}{2000}$ physostigmin sulphate. Since the crustaceans had

been taken from the same batch and were in approximately the same condition as those in the previous experiment, it was considered unnecessary to repeat the controls in the salts alone. The following is a table of the results:

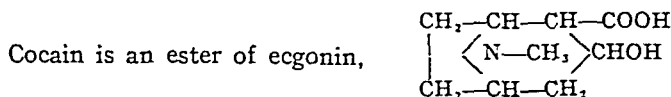
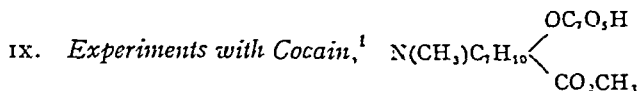
TABLE XVII.

Solution.	Toxicity.	Solution.	Toxicity.
$\frac{N}{12}$ $MgSO_4$	29×10^{-5}	$\frac{N}{12}$ $MgSO_4 + \frac{M}{2500}$ physos. sulph.	106×10^{-5}
$\frac{N}{12}$ $BaCl_2$	56×10^{-5}	$\frac{N}{12}$ $BaCl_2 +$ " " "	218×10^{-5}
$\frac{N}{12}$ CH_3COONa	14×10^{-5}	$\frac{N}{12}$ $CH_3COONa + \frac{M}{2500}$ " "	96×10^{-5}

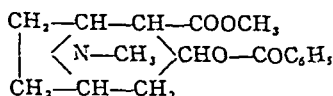
Subtracting the toxicity of the solution of the salt alone from that of the salt + alkaloid we have:

TABLE XVIII.

Salt.	Toxicity of $\frac{M}{2500}$ Physostigmin Sulphate + Salt — Toxicity of Salt.
$\frac{N}{12}$ $MgSO_4$	77×10^{-5}
" $BaCl_2$	162×10^{-5}
" CH_3COONa	82×10^{-5}



in which one hydrogen atom is replaced by a benzoyl group and another by a methyl group²; its constitution, therefore, is probably³



Cocain is a tertiary base and is very readily split up, even by boiling with water, into benzoyl-ecgonin and methyl alcohol.

¹ Beilstein, *loc. cit.*, iii, p. 866.

² Pictet, *loc. cit.*, pp. 234, 239, and 249.

³ Vide Cushny, *Pharmacology and Therapeutics*, p. 302, 1903.

(a) EXPERIMENTS WITH PARAMŒCIUM.

As in the experiments previously described, a small and constant measured amount of culture-fluid was placed in a paraffin cell of constant volume and the cell filled up with the solution, the action of which was to be tested. The cell was then immediately covered with a cover-glass so as to exclude air, the conditions as to oxygen, etc., being therefore practically constant. Any loss of fluid due to evaporation was supplied as soon as detected by placing a small drop of the solution at the edge of the cover-glass. In these experiments, however, the number of dead infusoria in the cell was counted at short intervals of time and the average duration of life was thus estimated. The toxicity of the solution was estimated as the reciprocal of the average duration of life. Controls in solutions of salts alone were made. From the nature of the experiments, they could not be done simultaneously so as to secure absolutely uniform conditions of temperature, etc. But they were performed within as short intervals of each other as possible. The temperature did not vary more than two or three degrees, variations which have practically no effect on the duration of life. The same culture was used invariably and the paramœcia were taken from the layer geotropically collected near the surface. The number of paramœcia in each experiment was not, of course, the same, but as the volume of culture was always the same, the modification of the toxicity of the solutions by impurities thus introduced, was probably the same for all. Since the number of infusoria in each experiment was not the same, each result is not of the same order of accuracy, still I believe that the number was always sufficient to exclude any appreciable error. Since it always took an appreciable period to complete the counting of the paramœcia, the *end* of that period was always estimated as the death-time. Thus, to quote the figures of an actual experiment, paramœcia were placed in $\frac{N}{90}$ MgSO_4 + $\frac{N}{10000}$ cocain hydrochloride:

In 3 minutes	2	were dead,	
" 5 "	3	more were dead,	
" 9 "	10	" " "	
" 13 "	29	" " "	
" 20 "	21	" " "	
" 26 "	15	" " "	
" 32 "	12	" " "	
" 39 "	10	" " "	
" 49 "	25	" " "	
" 60 "	15	" " "	
" 70 "	13	" " "	
" 75 "	3	" " "	
" 80 "	2	" " "	All dead.

Average duration of life, 34.3 minutes.

Toxicity of solution, 29.15×10^{-3} .

It should be pointed out that the errors due to the time occupied in counting become, actually, greater the greater the total number of dead, but at the same time, they are then of less importance inasmuch as they are less in proportion to the total period the estimation of which they affect

The following is a table of the results obtained:

TABLE XIX.

Solution	Number Infusoria in Cell	Toxicity	Solution	Number Infusoria in Cell	Toxicity
$\frac{N}{10}$ CaCl_2	20	Less than * 1.5×10^{-3}	$\frac{N}{10}$ CaCl_2 + $\frac{M}{10000}$ cocain hydrochloride	138	11.8×10^{-3}
* MgSO_4	18	1.1×10^{-3}	" MgSO_4 + " "	150	29.2×10^{-3}
* Na_2SO_4	35	5×10^{-3}	" Na_2SO_4 + " "	142	151.5×10^{-3}
* BaCl_2	90	70.4×10^{-3}	" BaCl_2 + " "	201	58.5×10^{-3}
* K_2SO_4	88	2×10^{-3}	" K_2SO_4 + " "	86	128.2×10^{-3}
* CH_3COONa	61	45×10^{-3}	" CH_3COONa + $\frac{M}{10000}$ "	391	27×10^{-3}
" CH_3COOK	55	1×10^{-3}	" CH_3COOK + " "	74	120.5×10^{-3}

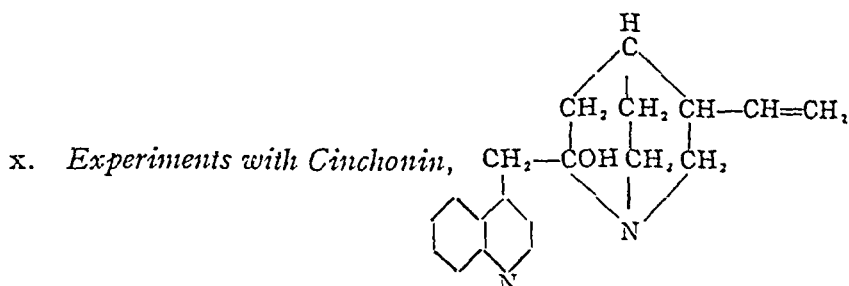
* The duration of life of the longest-lived individuals in CaCl_2 was not exactly determined.

Subtracting the toxicity of the salt alone from that of the salt + cocain hydrochloride so as to ascertain the influence of the salt upon the toxicity of the alkaloid itself, we have,

TABLE XX

Salt	Toxicity of $\frac{M}{10000}$ Cocain Hydrochloride + Salt — Toxicity of Salt
$\frac{N}{10}$ CaCl_2	10.3×10^{-3}
" MgSO_4	28.1×10^{-3}
" Na_2SO_4	151×10^{-3}
" BaCl_2	119×10^{-3}
" K_2SO_4	126.2×10^{-3}
" CH_3COONa	27.55×10^{-3}
" CH_3COOK	119.5×10^{-3}

Thus we see that in the case of BaCl_2 the addition of $\frac{M}{10000}$ cocain hydrochloride diminished the toxicity of the salt itself.



The structural formula of cinchonin is not quite certain, but the above is regarded by Pictet as the most probable. The hydrogen of the hydroxyl group can be replaced by benzoyl or acetyl. It is a somewhat strong diacid base.

(a) EXPERIMENTS WITH GAMMARUS.

In the same way as in the experiments with Gammarus previously described, ten individuals as uniform in size, condition, etc., as possible, from the same locality, and freed from adherent fluid with filter-paper, were placed in 52 c.c. each of the following solutions in finger-bowls: $\frac{N}{12}$ $MgSO_4$ + $\frac{N}{1368}$ cinchonin hydrochloride, $\frac{N}{82}$ $NaCl$ + $\frac{N}{1368}$ cinchonin hydrochloride, $\frac{N}{82}$ $BaCl_2$ + $\frac{N}{1368}$ cinchonin hydrochloride, $\frac{N}{82}$ NH_4Cl + $\frac{N}{1368}$ cinchonin hydrochloride, $\frac{N}{82}$ CH_3COONa + $\frac{N}{1368}$ cinchonin hydrochloride, $\frac{N}{82}$ CH_3COOK + $\frac{N}{1368}$ cinchonin hydrochloride, $\frac{N}{82}$ $MgSO_4$, $\frac{N}{82}$ $NaCl$, $\frac{N}{82}$ $BaCl_2$, $\frac{N}{82}$ NH_4Cl , $\frac{N}{82}$ CH_3COONa , $\frac{N}{82}$ CH_3COOK . The toxicities of the solutions were calculated as usual. The following is a table of the results obtained :

TABLE XXI

Solution.	Toxicity.	Solution	Toxicity.
$\frac{N}{12}$ $MgSO_4$	18×10^{-5}	$\frac{N}{12}$ $MgSO_4$ + $\frac{N}{1368}$ cinchonin hydrochloride	32×10^{-5}
$\frac{N}{82}$ $NaCl$	0×10^{-5}	$\frac{N}{82}$ $NaCl$ + " " "	40×10^{-5}
$\frac{N}{82}$ $BaCl_2$	77×10^{-5}	$\frac{N}{82}$ $BaCl_2$ + " " "	81×10^{-5}
$\frac{N}{82}$ NH_4Cl	134×10^{-5}	$\frac{N}{82}$ NH_4Cl + " " "	81×10^{-5}
$\frac{N}{82}$ CH_3COONa	21×10^{-5}	$\frac{N}{82}$ CH_3COONa + " " "	33×10^{-5}
$\frac{N}{82}$ CH_3COOK	314×10^{-5}	$\frac{N}{82}$ CH_3COOK + " " "	300×10^{-5}

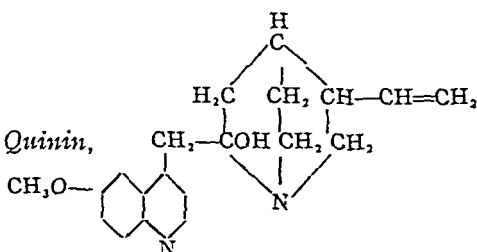
Subtracting, in the usual way, the toxicities of the salts alone from those of the salts + cinchonin, we have

TABLE XXII.

Salt.	Toxicity of $\frac{M}{1300}$ Cinchonin Hydrochloride + Salt — Toxicity of Salt.
$\frac{N}{2}$ MgSO ₄	14×10^{-5}
" NaCl	40×10^{-5}
" BaCl ₂	4×10^{-5}
" NH ₄ Cl	-53×10^{-5}
" CH ₃ COONa	12×10^{-5}
" CH ₃ COOK	5×10^{-5}

Thus in the case of ammonium chloride the addition of $\frac{M}{1300}$ cinchonin hydrochloride markedly diminished the toxicity of the salt for *Gammarus*.

x1. Experiments with Quinin,



Quinin is a diacid base and possesses a hydroxyl and a methoxyl group. The hydrogen of the hydroxyl group can be replaced by benzoyl and by acetyl.

(a) EXPERIMENTS WITH PARAMÆCIUM.

In the same way as in the experiments (a) with cocain, a small and constant measured amount of culture-fluid was mixed, under conditions of temperature, oxygen content, etc., as nearly constant as possible with a constant and measured volume of the solution to be tested. The experimental details were precisely the same as previously described, and the toxicities of the various solutions were calculated from the average duration of life in them.

The following is a table of the results obtained:

TABLE XXIII.

Solution.	Number of Infusoria in Cell.	Toxicity.	Solution.	Number of Infusoria in Cell.	Toxicity.
$\frac{N}{20}$ CaCl_2	20	Less than 1.5×10^{-3}	$\frac{N}{20}$ CaCl_2 + $\frac{M}{5000}$ quinin hydrochloride	120	27.3×10^{-3}
" MgCl_2	35	$.8 \times 10^{-3}$	" MgCl_2 + " " "	104	46.3×10^{-3}
" MgSO_4	18	1.1×10^{-3}	" MgSO_4 + " " "	30	48.8×10^{-3}
" Na_2SO_4	35	$.5 \times 10^{-3}$	" Na_2SO_4 + " " "	56	108.1×10^{-3}
" NaCl	36	$.55 \times 10^{-3}$	" NaCl + " " "	48	74.6×10^{-3}
" BaCl_2	90	70.4×10^{-3}	" BaCl_2 + " " "	—	About,* 66.7×10^{-3}
" NH_4Cl	—	18×10^{-3}	" NH_4Cl + " " "	40	24.75×10^{-3}
" K_2SO_4	88	2×10^{-3}	" K_2SO_4 + " " "	154	61.1×10^{-3}
" CH_3COONa	61	$.45 \times 10^{-3}$	" CH_3COONa + " " "	84	80×10^{-3}
" CH_3COOK	55	1×10^{-3}	" CH_3COOK + " " "	100	74×10^{-3}

* The duration of life of all the infusoria in $\frac{N}{20}$ BaCl_2 + $\frac{M}{5000}$ quinin hydrochloride was not exactly determined. The average duration of life was in this case estimated as the mean between the shortest and the longest death-times. Other experiments showed that this gives only a slightly too long duration of life when the average death-time is short—if it is long the inaccuracy is great. Probably the toxicity thus estimated for this solution is a little too low.

Subtracting, as usual, the toxicity of the salt alone from that of the salt + alkaloid we obtain the following table:

TABLE XXIV.

Salt.	Toxicity of $\frac{M}{5000}$ Quinin Hydrochloride + Salt — Toxicity of Salt.
$\frac{N}{20}$ CaCl_2	25.8×10^{-3}
" MgCl_2	45.5×10^{-3}
" MgSO_4	47.7×10^{-3}
" Na_2SO_4	107.6×10^{-3}
" NaCl	74.05×10^{-3}
" BaCl_2	-3.7×10^{-3}
" NH_4Cl	22×10^{-3}
" K_2SO_4	62.1×10^{-3}
" CH_3COONa	79.55×10^{-3}
" CH_3COOK	73×10^{-3}

(b) EXPERIMENTS WITH TUBIFEX.

As in the experiments with *Tubifex* previously described, 20 to 30 of the worms as alike as possible, from the same locality, and freed from adherent fluid, were placed in 55 c.c. of each of the following solutions: $\frac{N}{12}$ $MgCl_2$ + $\frac{N}{1500}$ quinin hydrochloride, $\frac{N}{12}$ $CaCl_2$ + $\frac{N}{1500}$ quinin hydrochloride, $\frac{N}{12}$ K_2SO_4 + $\frac{N}{1500}$ quinin hydrochloride, $\frac{N}{12}$ CH_3COONa + $\frac{N}{1500}$ quinin hydrochloride. The controls in $\frac{N}{12}$ salts alone were the same as in the experiments (c) with caffein (see p. 510).

In $\frac{N}{12}$ $MgCl_2$ + $\frac{N}{1500}$ quinin hydrochloride one or two motionless individuals were observed in 8 hrs. 10 min.; in 27 hrs. 20 min. there were still slight movements in a few; in 32 hrs. 20 min. all were motionless.

In $\frac{N}{12}$ $CaCl_2$ + $\frac{N}{1500}$ quinin hydrochloride no motionless individuals were observed up to 10 hrs. 30 min.; in 27 hrs. 20 min. the movements were slight; in 32 hrs. 20 min. all were motionless.

In $\frac{N}{12}$ K_2SO_4 + $\frac{N}{1500}$ quinin hydrochloride 5 were motionless in 3 hrs.; in 3 hrs. 50 min. 8 were motionless; in 6 hrs. 50 min. only a few were moving; in 10 hrs. 30 min. all were motionless.

In $\frac{N}{12}$ CH_3COONa + $\frac{N}{1500}$ quinin hydrochloride several were motionless in 5 hrs. 10 min.; in 5 hrs. 55 min. the worms were mostly motionless; in 8 hrs. 10 min. slight movements continued in one or two, the others being much disintegrated; in 10 hrs. 30 min. there were slight movements in one; in 27 hrs. 20 min. all were motionless.

Thus the order of toxicity of these salts plus quinin was: potassium sulphate, sodium acetate, magnesium chloride, calcium chloride. In the salts alone (see pp. 510, 517) the order of toxicity was potassium sulphate, calcium chloride, magnesium chloride, sodium acetate. This appears to indicate that sodium acetate and magnesium chloride favor the toxic action of quinin hydrochloride upon *Tubifex*.

(c) EXPERIMENTS WITH GAMMARUS.

(i) In the usual manner, ten individuals as alike as possible were placed in 52 c.c. of each of the following solutions in finger-bowls: $\frac{N}{12}$ $MgSO_4$ + $\frac{N}{1500}$ quinin hydrochloride, $\frac{N}{12}$ $NaCl$ + $\frac{N}{1500}$ quinin hydrochloride, $\frac{N}{12}$ $BaCl_2$ + $\frac{N}{1500}$ quinin hydrochloride, $\frac{N}{12}$ NH_4Cl + $\frac{N}{1500}$ quinin hydrochloride, $\frac{N}{12}$ CH_3COONa + $\frac{N}{1500}$ quinin hydrochloride, $\frac{N}{12}$ CH_3COOK + $\frac{N}{1500}$ quinin hydrochloride. The controls in salts alone were the same as in the experiments (a) with cinchonin (see p. 530).

The toxicities of the solutions were calculated as usual.

The following is a table of the results obtained:

TABLE XXV.

Solution	Toxicity	Solution	Toxicity.
$\frac{N}{52} \text{MgSO}_4$	18×10^{-5}	$\frac{N}{52} \text{MgSO}_4 + \frac{M}{1250} \text{quinin hydrochloride}$	27×10^{-5}
$\frac{N}{52} \text{NaCl}$	0×10^{-5}	$\frac{N}{52} \text{NaCl} + \text{ " " " "}$	45×10^{-5}
$\frac{N}{52} \text{BaCl}_2$	77×10^{-5}	$\frac{N}{52} \text{BaCl}_2 + \text{ " " " "}$	68×10^{-5}
$\frac{N}{52} \text{NH}_4\text{Cl}$	134×10^{-5}	$\frac{N}{52} \text{NH}_4\text{Cl} + \text{ " " " "}$	86×10^{-5}
$\frac{N}{52} \text{CH}_3\text{COONa}$	21×10^{-5}	$\frac{N}{52} \text{CH}_3\text{COONa} + \text{ " " " "}$	38×10^{-5}
$\frac{N}{52} \text{CH}_3\text{COOK}$	314×10^{-5}	$\frac{N}{52} \text{CH}_3\text{COOK} + \text{ " " " "}$	247×10^{-5}

Subtracting, as usual, the toxicities of the salts alone from those of the salts + quinin, we have:

TABLE XXVI.

Salt.	Toxicity of $\frac{N}{1350} \text{Quinin Hydrochloride} + \text{Salt}$ — Toxicity of Salt.
$\frac{N}{52} \text{MgSO}_4$	9×10^{-5}
" NaCl	45×10^{-5}
" BaCl ₂	-9×10^{-5}
" NH ₄ Cl	-48×10^{-5}
" CH ₃ COONa	17×10^{-5}
" CH ₃ COOK	-67×10^{-5}

So that in three cases, namely, in barium chloride, ammonium chloride, and potassium acetate, the addition of $\frac{N}{1350}$ quinin hydrochloride reduced the toxicity of the salt in a more or less marked degree.

(ii) Ten individuals as alike as possible, etc., were placed in 52 c. c. of each of the following solutions:

- XIII 50 c. c. $\frac{N}{10} \text{Na}_2\text{HPO}_4 + 2 \text{ c. c. } \frac{N}{50} \text{quinin hydrochloride.}$
 XIV 40 c. c. $\frac{N}{10} \text{Na}_2\text{HPO}_4 + 10 \text{ c. c. } \frac{N}{10} \text{NaH}_2\text{PO}_4 + 2 \text{ c. c. } \frac{N}{50} \text{quinin hydrochloride.}$
 XV 30 c. c. $\frac{N}{10} \text{Na}_2\text{HPO}_4 + 20 \text{ c. c. } \frac{N}{10} \text{NaH}_2\text{PO}_4 + 2 \text{ c. c. } \frac{N}{50} \text{quinin hydrochloride.}$
 XVI 20 c. c. $\frac{N}{10} \text{Na}_2\text{HPO}_4 + 30 \text{ c. c. } \frac{N}{10} \text{NaH}_2\text{PO}_4 + 2 \text{ c. c. } \frac{N}{50} \text{quinin hydrochloride.}$
 XVII 10 c. c. $\frac{N}{10} \text{Na}_2\text{HPO}_4 + 40 \text{ c. c. } \frac{N}{10} \text{NaH}_2\text{PO}_4 + 2 \text{ c. c. } \frac{N}{50} \text{quinin hydrochloride.}$
 XVIII 50 c. c. $\frac{N}{10} \text{NaH}_2\text{PO}_4 + 2 \text{ c. c. } \frac{N}{50} \text{quinin hydrochloride.}$

The controls with salts alone were the same as in the experiments (d,ii) with caffeine (see p. 512).

The following is a table of the results obtained. The Roman numerals I, II, etc., up to VI refer to the solutions of salts alone as in Tables III and IV while the numerals XIII, XIV, etc., to XVIII refer to the solutions the composition of which has just been described.

TABLE XXVII.

Solution.	Toxicity	Solution.	Toxicity.
I	123×10^{-5}	XIII	226×10^{-5}
II	139×10^{-5}	XIV	125×10^{-5}
III	112×10^{-5}	XV	88×10^{-5}
IV	85×10^{-5}	XVI	94×10^{-5}
V	89×10^{-5}	XVII	96×10^{-5}
VI	107×10^{-5}	XVIII	123×10^{-5}

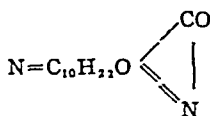
Subtracting the toxicities of the salts alone from those of the salts + quinin, we have:

TABLE XXVIII.

Saline Solution	Toxicity of $\frac{M}{125}$ Quinin Hydrochloride + Saline Solution— Toxicity of Saline Solution.
I	103×10^{-5}
II	-14×10^{-5}
III	-24×10^{-5}
IV	9×10^{-5}
V	7×10^{-5}
VI	16×10^{-5}

Thus in two cases, namely, in Solutions II and III, the addition of $\frac{M}{1300}$ quinin hydrochloride diminished the toxicity of the saline mixture itself for *Gammarus*.

XII. Experiments with Strychnin,



The constitution of strychnin is very imperfectly known.

There is some reason, however, to suspect the presence of a hydroxyl group, although strychnin is unaffected by acetic anhydride. No oxymethyl group is present. Strychnin is a monacid base, forming salts with only one molecule of acid, although it contains two nitrogen atoms in the molecule.

(a) PREVIOUS INVESTIGATIONS.

Zoethout¹ has found that sodium hydroxide markedly increases the toxicity of strychnin for paramœcia, while hydrochloric acid does so to a less extent. Fischer² was unable to detect any distinct influence of calcium chloride, barium chloride, magnesium chloride, or sodium citrate upon the toxicity of strychnin for rabbits or frogs.

(b) EXPERIMENTS WITH PARAMŒCIUM.

As usual in the experiments with paramœcium, a measured and constant amount of culture-medium was placed in a measured and constant volume of the solution under investigation. The conditions of the experiment were the same as those previously described (p. 528).

In $\frac{N}{80}$ Na_2SO_4 + $\frac{N}{10000}$ strychnin nitrate, several disintegrated individuals were noted in 3 min.; in 5 min. many, and in 10 min. nearly all, were dead; in 18 min., all were dead.

In $\frac{N}{80}$ BaCl_2 + $\frac{N}{10000}$ strychnin nitrate, many were dead in two min.; in 10 min., all were dead.

Taking the average duration of life to be the mean between the shortest and longest death-times, a method of estimating the death-time which is fairly accurate for short periods (see foot-note p. 532), we obtain the following results:

TABLE XXIX.

Solution.	Toxicity.	Solution.	Toxicity.
$\frac{N}{80}$ Na_2SO_4	$.5 \times 10^{-3}$ *	$\frac{N}{80}$ Na_2SO_4 + $\frac{N}{10000}$ strychnin nitrate	95.2×10^{-3}
$\frac{N}{80}$ BaCl_2	70.4×10^{-3} *	$\frac{N}{80}$ BaCl_2 + " " "	166.7×10^{-3}

* See p. 532.

This would give a difference between the toxicity of the salt + strychnin and that of the salt alone of 94.7×10^{-3} in the case of Na_2SO_4 and of 96.3×10^{-3} in the case of BaCl_2 . Too much reliance must not be placed on these figures, however, since a considerable interval of time separated these experiments with strychnin from those in which the toxicities of

¹ *Amer. Journ. of Physiol.*, ii, p. 220, 1899.

² *Ibid.*, x, p. 345, 1904.

the salts alone for paramœcia were determined, and besides, the method of calculating the toxicity from the mean of the shortest and the longest death-times is inaccurate, and the degree of inaccuracy obviously depends on a variety of factors.

(c) EXPERIMENTS WITH GAMMARUS.

Five individuals were placed in the usual way and with the usual precautions in 55 c. c. each of the following solutions: $\frac{N}{12}$ $MgSO_4$ + $\frac{M}{1300}$ strychnin nitrate, $\frac{N}{12}$ Na_2SO_4 + $\frac{M}{1300}$ strychnin nitrate, $\frac{N}{12}$ $NaCl$ + $\frac{M}{1300}$ strychnin nitrate, $\frac{N}{12}$ $BaCl_2$ + $\frac{M}{1300}$ strychnin nitrate, $\frac{N}{12}$ NH_4Cl + $\frac{M}{1300}$ strychnin nitrate, $\frac{N}{12}$ CH_3COONa + $\frac{M}{1300}$ strychnin nitrate, $\frac{N}{12}$ CH_3COOK + $\frac{M}{1300}$ strychnin nitrate. The controls in salts alone were the same as in the experiments (c i) with pilocarpin (Table, p. 522).

The following is a table of the results obtained :

TABLE XXX.

Solution.	Toxicity.	Solution.	Toxicity.
$\frac{N}{12}$ $MgSO_4$	29×10^{-5}	$\frac{N}{12}$ $MgSO_4$ + $\frac{M}{1300}$ strychnin nitrate	35×10^{-5}
" Na_2SO_4	68×10^{-5}	" Na_2SO_4 + " " "	59×10^{-5}
" $NaCl$	5×10^{-5}	" $NaCl$ + " " "	35×10^{-5}
" $BaCl_2$	56×10^{-5}	" $BaCl_2$ + " " "	69×10^{-5}
" NH_4Cl	106×10^{-5}	" NH_4Cl + " " "	70×10^{-5}
" CH_3COONa	14×10^{-5}	" CH_3COONa + $\frac{M}{1300}$ " "	229×10^{-5}
" CH_3COOK	518×10^{-5}	" CH_3COOK + " " "	316×10^{-5}

Subtracting the toxicities of the salts from those of the salts + strychnin we have:

TABLE XXXI.

Salt.	Toxicity of $\frac{M}{1300}$ Strychnin Nitrate + Salt — Toxicity of Salt.
$\frac{N}{12}$ $MgSO_4$	6×10^{-5}
" Na_2SO_4	— 9×10^{-5}
" $NaCl$	35×10^{-5}
" $BaCl_2$	13×10^{-5}
" NH_4Cl	— 36×10^{-5}
" CH_3COONa	215×10^{-5}
" CH_3COOK	— 202×10^{-5}

Thus in three cases, namely in sodium sulphate, ammonium chloride, and potassium acetate, the addition of $\frac{M}{1300}$ strychnin nitrate diminished the toxicity of the salt itself to a more or less marked degree.

XIII. *Experiments with Veratrin*, $C_{27}H_{41}NO_3$ — $O-CO-C \begin{array}{l} \nearrow \text{CH}_3 \\ \searrow \text{CH}-CH_3 \end{array}$

The constitution of veratrin is very imperfectly known; it is a tertiary base and contains no methoxyl group; it dissolves in dilute acids and in an excess of caustic alkali with which it forms salts.

(a) PREVIOUS INVESTIGATIONS.

In 1885 Ringer¹ found that veratrin is similar to calcium salts as regards its action upon the ventricle of the frog's heart, and that veratrin can be antagonized by potassium chloride just as calcium chloride can; he did not find, however, as the title of his paper would appear to indicate, that calcium salts antagonized veratrin and, as I shall show later, such is not the case. The antagonism between veratrin and potassium chloride was confirmed for the embryonic heart of the chicken by Pickering,² who found, however, that, whereas potassium chloride antagonized veratrin, the antagonism is not *mutual* in the sense in which Langley has defined the term,³ for veratrin will not antagonize potassium chloride. This antagonism between veratrin and potassium chloride has been established for the striated muscles of the frog by Buchanan,⁴ who also confirmed Biedermann's statement⁵ that sodium chloride assists the veratrin effect. Zoethout⁶ has shown that sodium hydroxide increases the toxicity of veratrin for paramœcia.

(b) EXPERIMENTS WITH PARAMŒCIUM.

Under the conditions previously described, a known and constant

¹ *Journ. of Physiol.*, v, p. 352.

² *Ibid.*, xiv, p. 383.

³ *Ibid.*, i, p. 339, 1878.

⁴ *Ibid.*, xxv, p. 137.

⁵ *Electrophysiologie*, p. 93.

⁶ *Amer. Journ. of Physiol.*, ii, p. 220.

volume of culture-medium was acted upon by a known and constant volume of the solution which was being tested.

In $\frac{N}{25}$ Na_2SO_4 + $\frac{N}{10000}$ veratrin sulphate, many were dead in 3 min.; in 5 min., most of the infusoria were dead, and in 15 min. all were dead.

In $\frac{N}{25}$ BaCl_2 + $\frac{N}{10000}$ veratrin sulphate, several were dead in 3 min.; in 5 min. many, and in 10 min. all.

In $\frac{N}{25}$ MgCl_2 + $\frac{N}{10000}$ veratrin sulphate, the organisms still appeared to be in a normal condition at the end of 25 min.

In $\frac{N}{25}$ MgSO_4 + $\frac{N}{10000}$ veratrin sulphate, the organisms appeared to be still in a normal condition at the end of 20 min.

Estimating the average duration of life in these solutions as the mean between the shortest and the longest death-times, and using the figures previously obtained (see p. 532) for the toxicities of the salts alone, we have the following results

TABLE XXXII.

Solution	Toxicity	Solution	Toxicity
$\frac{N}{25}$ MgCl_2	8×10^{-3}	$\frac{N}{25}$ MgCl_2 + $\frac{N}{10000}$ veratrin sulphate	less than 40×10^{-3}
$\frac{N}{25}$ MgSO_4	1.1×10^{-3}	$\frac{N}{25}$ MgSO_4 + " "	less than 50×10^{-3}
$\frac{N}{25}$ Na_2SO_4	5×10^{-3}	$\frac{N}{25}$ Na_2SO_4 + " "	111.1×10^{-3}
$\frac{N}{25}$ BaCl_2	70.4×10^{-3}	$\frac{N}{25}$ BaCl_2 + " "	153.8×10^{-3}

Subtracting the toxicities of the salts alone from those of the salts + veratrin, we have:

TABLE XXXIII.

Salt	Toxicity of $\frac{N}{10000}$ Veratrin Sulphate + Salt — Toxicity of Salt
$\frac{N}{25}$ MgCl_2	Less than 39.2×10^{-3}
" MgSO_4	Less than 48.9×10^{-3}
" Na_2SO_4	110.6×10^{-3}
" BaCl_2	83.4×10^{-3}

These figures require accepting with the same caution and reservations as those which were obtained in the experiments on *Paramacium* with strychnin (see p. 536).

(c) EXPERIMENTS WITH GAMMARUS.

(2) Five individuals were placed in the usual way and with the usual precautions in 52 c. c. each of the following solutions: $\frac{N}{25}$ MgSO_4 + $\frac{N}{10000}$ veratrin sulphate, $\frac{N}{25}$ BaCl_2 + $\frac{N}{10000}$ veratrin sulphate, $\frac{N}{25}$ K_2SO_4 + $\frac{N}{10000}$ veratrin sulphate, $\frac{N}{25}$ CH_3COONa + $\frac{N}{10000}$ veratrin sulphate. The controls

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with salts alone were the same as those in the experiments (c, d) with pilocarpin (Table XI, p. 522).

The following is a table of the results obtained:

TABLE XXXIV.

Solution.	Toxicity	Solution	Toxicity
$\frac{N}{52} \text{MgSO}_4$	29×10^{-5}	$\frac{N}{52} \text{MgSO}_4 + \frac{M}{80000} \text{veratrin sulphate}$	303×10^{-5}
" BaCl_2	56×10^{-5}	" $\text{BaCl}_2 + \text{ " " "}$	67×10^{-5}
" K_2SO_4	372×10^{-5}	" $\text{K}_2\text{SO}_4 + \text{ " " "}$	455×10^{-5}
" CH_3COONa	14×10^{-5}	" $\text{CH}_3\text{COONa} + \text{ " " "}$	124×10^{-5}

Subtracting the toxicities of the salts alone from those of the salts + veratrin, we have the following results:

TABLE XXXV.

Salt.	Toxicity of $\frac{M}{80000} \text{Veratrin Sulphate} + \text{Salt} - \text{Toxicity of Salt.}$
$\frac{N}{52} \text{MgSO}_4$	274×10^{-5}
" BaCl_2	11×10^{-5}
" K_2SO_4	83×10^{-5}
" CH_3COONa	110×10^{-5}

(ii) This experiment was repeated with five more individuals from the same batch as before; since they were in approximately the same condition as those of the previous experiment, it was considered unnecessary to repeat the controls. Five crustaceans were placed in 52 c. c. of each of the following solutions: $\frac{N}{52} \text{MgSO}_4 + \frac{M}{80000} \text{veratrin sulphate}$, $\frac{N}{52} \text{BaCl}_2 + \frac{M}{80000} \text{veratrin sulphate}$, $\frac{N}{52} \text{CH}_3\text{COONa} + \frac{M}{80000} \text{veratrin sulphate}$.

The following is a table of the results:

TABLE XXXVI.

Solution.	Toxicity.	Solution.	Toxicity.
$\frac{N}{52} \text{MgSO}_4$	29×10^{-5}	$\frac{N}{52} \text{MgSO}_4 + \frac{M}{80000} \text{veratrin sulphate}$	95×10^{-5}
" BaCl_2	56×10^{-5}	" $\text{BaCl}_2 + \text{ " " "}$	80×10^{-5}
" CH_3COONa	14×10^{-5}	" $\text{CH}_3\text{COONa} + \text{ " " "}$	85×10^{-5}

Subtracting the toxicities of the salts alone from those of the salts + veratrin, we have the following results:

TABLE XXXVII.

Salt	Toxicity of $\frac{N}{10000}$ Veratrin Sulphate + Salt — Toxicity of Salt.
$\frac{N}{100} \text{MgSO}_4$	66×10^{-5}
" BaCl_2	24×10^{-5}
" CH_3COONa	71×10^{-5}

(d) EXPERIMENTS WITH THE FROG'S GASTROCNEMIUS.

(i) Placed the gastrocnemius of the left leg in a watch glass and covered it with a solution (1) made up of 100 c.c. $\frac{N}{10} \text{NaCl}$ + 5 c.c. $\frac{N}{10} \text{CaCl}_2$ + $\frac{N}{100000}$ veratrin sulphate, and that of the right leg in solution (2) made up of 100 c.c. $\frac{N}{10} \text{NaCl}$ + 5 c.c. $\frac{N}{10} \text{Na}_2\text{SO}_4$ + $\frac{N}{100000}$ veratrin sulphate and stimulated the sciatic nerve of each in 4 minutes. The muscle in solution (1) responded by a typical long veratrin contraction, while that in (2) responded by a normal *twitch* both on make and on break—this continued during 7 minutes, during which the muscles were each stimulated twice—then the muscle in (2) gave the veratrin contraction as well and this continued to be the case after $3\frac{1}{2}$ hours. It was then noticed that in the muscle in (2) the veratrin effect was very much more easily fatigued than in the muscle in solution (1), *i. e.* if both were stimulated and then stimulated again within one minute, the muscle in (1) still gave a typical veratrin contraction, while that in (2) only gave a twitch. After 6 hours (2) gave only twitches on excitation, while (1) gave a weak veratrin contraction.

Thus it would appear not only that calcium chloride favors the veratrin effect but that sodium sulphate diminishes it. Of course this may be simply due to the removal of calcium from the tissues by the sodium sulphate.¹

(ii) Placed the gastrocnemius of the right leg in a watch glass and covered with a solution (1) made up of 100 c.c. $\frac{N}{10} \text{NaCl}$ + 5 c.c. $\frac{N}{10} \text{BaCl}_2$ + $\frac{N}{100000}$ veratrin sulphate, and that of the right leg in (2) made up of 100 c.c. $\frac{N}{10} \text{NaCl}$ + 5 c.c. $\frac{N}{10} \text{KCl}$ + $\frac{N}{100000}$ veratrin sulphate. In 4 minutes both gave veratrin contractions, but that in (1) was much the greater. In 20 min. the muscle in (1) gave a prolonged veratrin contraction while that in (2) gave only irregular twitches. Myograms taken after 1 hour showed traces of a veratrin effect in the prolongation of the relaxation phase in the contraction of the muscle in (2), but the muscle in (1) showed a prolonged and normal veratrin contraction of about 4 times the height of that of the muscle in (2).

(iii) Placed the right gastrocnemius in (1) 100 c.c. $\frac{N}{10} \text{NaCl}$ + 5 c.c.

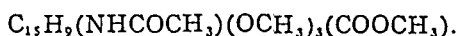
¹ Vide Loeb, *Amer. Journ. of Physiol.*, v, p. 362, 1901.

$\frac{N}{10}$ BaCl₂ + $\frac{M}{100000}$ veratrin sulphate, and the left gastrocnemius in (2) 100 c. c. $\frac{N}{10}$ NaCl + 5 c. c. $\frac{N}{10}$ Na₂SO₄ + $\frac{M}{100000}$ veratrin sulphate. The muscle in (1) on stimulation in 7 min. gave a prolonged veratrin contraction lasting about one minute (estimate), while that in (2) only gave a short veratrin contraction lasting about 10 seconds (estimate). After 1½ hours myograms showed traces of a veratrin effect in the muscle in (2) but the contraction was only one-sixth of the height of that in (1).

(iv) Placed right gastrocnemius in solution (1) 100 c. c. $\frac{N}{10}$ NaCl + 5 c. c. $\frac{N}{10}$ CH₃COOK + $\frac{M}{100000}$ veratrin, and left gastrocnemius in (2) 100 c. c. $\frac{N}{10}$ NaCl + 5 c. c. $\frac{N}{10}$ MgCl₂ + $\frac{M}{100000}$ veratrin sulphate. The muscle in (1) only gave a simple twitch on stimulation in 3, 6, 10, and 15 minutes, while that in (2) gave a slightly prolonged twitch in 3 minutes, and in 5 minutes a much prolonged contraction, and the same in 10 and 15 minutes. After 1 hour the muscle in (1) still only gave a twitch, while that in (2) gave a true veratrin contraction.

Control experiments with salts alone showed that these effects could not be due simply to the action of the salts themselves on the muscle, since the differences between the myograms after exposure to the same solutions without the veratrin were very much less than the differences recorded above. Hence it would appear that calcium chloride, magnesium chloride, and barium chloride tend to favor the veratrin effect in striated muscle, while sodium sulphate, potassium chloride, and potassium acetate tend to antagonize it.

XIV. *Experiments with Colchicin,*



The constitution of colchicin is imperfectly known; it possesses basic properties, but it is the methyl ester of a monobasic acid colchicein, C₂₀H₂₂NO₄(COOH), and is readily decomposed with the formation of the acid and methyl alcohol.

(a) EXPERIMENTS WITH GAMMARUS.

Five of the crustaceans under the usual conditions and with the usual precautions were placed in 52 c. c. each of the following solutions: $\frac{N}{2}$ MgSO₄ + $\frac{M}{100000}$ colchicin, $\frac{N}{2}$ BaCl₂ + $\frac{M}{100000}$ colchicin, $\frac{N}{2}$ K₂SO₄ + $\frac{M}{100000}$ colchicin, $\frac{N}{2}$ CH₃COONa + $\frac{M}{100000}$ colchicin. The controls in salts alone were the same as those in the experiments (c 2) with pilocarpin (Table XI, p. 522). The following is a table of the results:

TABLE XXXVIII.

Solution	Toxicity	Solution	Toxicity
$\frac{N}{52} \text{MgSO}_4$	29×10^{-5}	$\frac{N}{52} \text{MgSO}_4 + \frac{M}{10000} \text{colchicin}$	42×10^{-5}
" BaCl_2	56×10^{-5}	" $\text{BaCl}_2 + \text{ " "}$	126×10^{-5}
" K_2SO_4	372×10^{-5}	" $\text{K}_2\text{SO}_4 + \text{ " "}$	369×10^{-5}
" CH_3COONa	14×10^{-5}	" $\text{CH}_3\text{COONa} + \frac{M}{10000} \text{ "}$	36×10^{-5}

Subtracting, as usual, the toxicities of the salts alone from those of the salts + colchicin, we have the following results:

TABLE XXXIX.

Salt	Toxicity of $\frac{M}{10000} \text{Colchicin} + \text{Salt} - \text{Toxicity of Salt}$
$\frac{N}{52} \text{MgSO}_4$	13×10^{-5}
" BaCl_2	70×10^{-5}
" K_2SO_4	-3×10^{-5}
" CH_3COONa	22×10^{-5}

We see that the addition of $\frac{M}{10000}$ colchicin to the $\frac{N}{52}$ potassium sulphate slightly diminished its toxicity.

In regard to the chemicals used in these experiments, the caffeine, nicotin, pilocarpin, physostigmin sulphate, strychnin nitrate, and veratrin sulphate were Merck's C. P. The leucin and quinolin were Kahlbaum's, C. P. The quinin hydrochloride had been manufactured by Power and Weightman, while the cinchonin hydrochloride was kindly placed at my disposal by Dr. H. C. Biddle. The theobromin and colchicin were supplied by Lengfeld's pharmacy in San Francisco. The MgSO_4 , BaCl_2 , NH_4Cl , CH_3COONa , and CH_3COOK were Merck's C. P., the CaCl_2 and NaCl were Baker & Adamson's C. P., the Na_2SO_4 and K_2SO_4 were Kahlbaum's C. P., and the MgCl_2 was manufactured by de Haën.

III. THEORETICAL.

(1) *The Evidence that Alkaloids Combine with Some Constituent of Protoplasm.*

The idea that the toxic action of alkaloids depends upon their power of combining with some constituent of the proto-

plasm is by no means a new one. It was suggested independently by Langley¹ in 1878 and by Ringer and Morshead² in 1879. The evidence upon which Langley based the hypothesis was the distinctly local and selective action of many of the alkaloids, seeming to imply the existence of some substance or substances in the regions selected which have a particular affinity for the drug. The antagonism between atropin and pilocarpin was held to be due to one of the alkaloids displacing the other from its combination with the protoplasm by some form of mass-action. Ringer and Morshead were led to their opinion, on the other hand, by the fact that atropin and muscarin, although both paralyze cardiac muscle, nevertheless antagonize one another when both are made to act on the heart. They conjectured, as Langley did, that the one alkaloid displaced the other from its combination; they considered that atropin, from its stronger affinity for the tissues affected, displaces the muscarin, but as its paralyzing action is slight, the heart beats again.

Analytical evidence in favor of the hypothesis of chemical combination between alkaloids and tissues for which they have, especial affinity was afforded in 1888 by the investigations of Lovett,³ who showed that strychnin, after administration, is mainly stored up in the spinal cord—the cord containing a higher percentage of the alkaloid than any other organ or the blood itself, while in 1892 Loew⁴ demonstrated that caffein combines directly with certain portions of the protoplasm. Hamburger⁵ states that it is extremely probable that the alkaloids combine with some proteid constituent of the cell.

The hypothesis of chemical combination between alkaloids and some constituent of protoplasm therefore rests upon a fairly sound basis of physiological and of direct—that is to say, chemical—evidence. It is true that we have not obtained direct chemical evidence that every toxic alkaloid combines with the tissues it most affects, but the physiological evidence, supported

¹ *Journ. of Physiol.*, i, p. 339.

² *Ibid.* ii, p. 235; see also Ringer, *ibid.*, ii, p. 436.

³ *Ibid.*, ix, p. 99.

⁴ *Notice sur le Deuxième Congrès de Physiologie*, Liège, p. 32, 1892—quoted after Pickering, *Journ. of Physiol.*, xiv, p. 383.

⁵ *Osmotischer Druck und Ionenlehre*, iii, p. 252, 1904.

by such chemical evidence as we have, appears to render it a very fair assumption.

(2) *That the Toxicity of an Alkaloid for a Tissue is Influenced by the Ions which are also Acting upon it.*

I have already, in describing the experiments, alluded to many instances of this influence which were previously known, while my own experiments add to the number. Other investigations in this direction, to which I have not previously alluded, are those of Brown and Neilson¹ who have found that, using equimolecular solutions of different salts of the same alkaloid, the intensity of the action of the alkaloid is modified by the anion with which it is combined. Fujitani,² in studying the influence of various salts of morphin and of caffein upon the course of artificial digestion, has reached the same conclusion. This may, of course, be due to the different degree of dissociation of the different salts. On the other hand, it appears possible that it may be due to the different proportions of the hydrogen ions and anions set free by hydrolytic dissociation entering the tissue and altering it chemically, for, since the anions of the different acids set free by hydrolytic dissociation have different velocities of migration, the ratio of anions to hydrogen ions entering the tissue upon this account might be expected to be different with different salts of the alkaloid.

(3) *That the Antagonism between Different Alkaloids and between Alkaloids and Salts Depends upon the Tissue.*

That true antagonism between drugs does exist may be regarded as certain—the objections formerly raised by Rossbach³ having been satisfactorily answered by Langley,⁴ Prevost,⁵ Ringer,⁶ and others.

That the antagonism between different alkaloids or between alkaloids and salts is not a chemical reaction depending solely

¹ *American Journ. of Physiol.*, xiii, p. 427.

² *Arch. internat. d. Pharmacodyn. et d. Ther.*, xiv, p. 1, 1905.

³ Plüger's *Arch. f. d. ges. Physiol.*, xxi, 1879.

⁴ *Journ. of Anat. and Physiol.*, x, p. 187, 1875; xi, p. 173; *Journ. of Physiol.*, i, p. 320, 1878; iii, p. 11.

⁵ *Arch. d. physiol. norm. et path.*, deuxième Ser., iv, p. 801, 1877.

⁶ Ringer and Morshead, *Journ. of Physiol.*, ii, p. 235. Ringer, *ibid.*, v, p. 246.

on the antagonistic substances as factors is shown by several facts.

In the first place, although it may well be true in some cases, there is no reason to suppose that a direct chemical reaction takes place between the antagonistic substances themselves,—between pilocarpin and atropin, between muscarin and atropin, between physostigmin and nicotin,¹ for example,—and between a variety of neutral salts and alkaloids. The experiments which I have described show that every neutral salt, the action of which was tested, exerted some influence upon the toxicity of the alkaloid under consideration. It certainly appears inadmissible to assume a direct chemical interaction between each of these pairs of substances.

In the second place, it is not possible to find a mixture, for example, of atropin and pilocarpin which will produce practically no effect or a different effect from the original substances, as we should expect it to be if the antagonism between atropin and pilocarpin is “between themselves”—in fact, a mixture can be made which in small doses shows a pilocarpin action and in large doses an atropin action.²

In the third place, the antagonism is not always mutual, as we should expect it to be if it did not depend on the tissue. Thus, potassium chloride will antagonize the action of veratrin upon the embryonic heart, but veratrin will not antagonize potassium chloride.³ It is very probable, therefore, that the influence of another alkaloid or of a salt upon the toxic action of an alkaloid depends upon a chemical reaction in which the alkaloid, the other alkaloid or the salt, and some constituent, possibly proteid, of the tissue form a system of at least three reacting substances.

(4) *Measure of the Possible Acidity or Basicity of the Ion-proteids in a Tissue.*

I have already, in the introduction, alluded briefly to the reasons for suggesting the hypothesis that the acid or basic characters of the ion-proteids may be influenced by the acid or basic character of the ion in combination. A rather fuller treatment

¹ Zalackas, *Compt. rend. d. l'Acad. d. Sc.*, cxl, p. 741, 1905.

² Marshall, *Journ. of Physiol.*, xxxi, p. 120, 1904.

³ Pickering, *loc. cit.*

of the question will be found in the paper in the preceding number of this journal, while the experiments, therein described, on the influence of electrolytes upon the staining of tissues in methyl green and in iodine-eosin afford, in general, the results indicated by the hypothesis.

Assuming that ions which are basic in character, such as metal or hydrogen ions, tend to enhance the *basic* characters of the ion-proteid molecule, as the basic characters of phosphine are enhanced by the substitution of methyl groups for hydrogen atoms, and that ions which are acid in character, such as acid radicals or hydroxyl ions tend to enhance the *acid* characters of the ion-proteid molecule, as the acid characters of phenol are enhanced by the substitution of NO_3 groups for hydrogen atoms, we should expect the mass of anions or of kations entering a tissue to afford a measure of the acidity or basicity, respectively, of the ion-proteids—provided, that is, that the ions present in the greater mass take the greater share of the proteid. The number of anions diffusing across a given area in a given time is proportional to Hittorf's transport-number for the anion ($\frac{v}{u+v}$), provided that the ions diffusing across the area do not thereby give rise to a potential difference on the two sides of the area—a condition that is here satisfied, for it is assumed that the ions combine with proteid on entering the tissue. Similarly the transport-number for the kation ($\frac{u}{u+v}$) provides a measure of the mass of kations diffusing across a given area in a given time— u and v being the velocities of the kation and of the anion respectively. Thus, *ex hypothesi*, the mass of anions entering a tissue, and therefore the acidity of the ion-proteids formed, is proportional to $\frac{v}{u+v}$ for the electrolyte under consideration, while the basicity is proportional to $\frac{u}{u+v}$.

Let A be proportional to the number of ion-proteid molecules of acid character, so that $A = \frac{v}{u+v}$; and let B be similarly proportional to the number of basic ion-proteid molecules, then:

$$A - B = \frac{v-u}{u+v} = 1 - \frac{2u}{u+v}$$

$$\therefore A - B = 2(0.5 - \frac{u}{u+v})$$

Hence, if $\frac{u}{u+v}$ be greater than 0.5,—that is, if u be greater than v , we should expect the resulting ion-proteid to be basic in character, while if $\frac{u}{u+v}$ be less than 0.5—that is, if $\frac{v}{u+v}$ be greater

than 0.5, we should expect the resulting ion-proteid to be acid in character.

The following is a table of the transport-numbers for the anion of the electrolytes (except the phosphates) used in the experiments described in this paper (the transport-number for the kation is, of course, obtained from that for the anion by subtracting it from unity).

The figures here tabulated are all taken from Hittorf's results for dilute solutions quoted by Fitzpatrick.¹ In every case the number for the least concentration quoted is taken—the concentration varying between about .01 and 0.1 equivalent gram-molecules per liter.

TABLE XL.

Electrolyte	Transport-Number	Electrolyte	Transport-Number.
CaCl ₂	.683	BaCl ₂	.61
MgCl ₂	.678	NH ₄ Cl	.508
MgSO ₄	.656	K ₂ SO ₄	.498
Na ₂ SO ₄	.634	CH ₃ COONa	.443
NaCl	.622	CH ₃ COOK	.324

(5) *The Relation between the Transport-numbers for the Ions of an Electrolyte and the Toxicity of an Alkaloid in a Solution of the Electrolyte.*

In the curves (Plates II-IV, Figs. 1-4 and 6-7) the abscissæ measured to the right of the origin are proportional to $\frac{u}{u+v}$ for the electrolyte under consideration, while to the left of the origin they are proportional to $\frac{v}{u+v}$. In other words, the abscissæ, considered positive when measured to the *right* of the origin, are proportional to $0.5 - \frac{v}{u+v}$; that is, in the terms of the hypothesis to B-A. The ordinates are proportional to the toxicity of the alkaloid in a solution of the electrolyte as given in the tables in the account of the experiments.

It will be observed that the curves for caffein (Fig. 1), for

¹ *British Association Report*, 1893, Reprinted by Whetham in his *Theory of Solution and Electrolysis*, 1902.

quinolin (Fig. 2), and for leucin (Fig. 2.) each show one distinct maximum of toxicity—in each case this maximum occurs when the alkaloid is acting in a solution of potassium sulphate ($\frac{N}{50}$). The curves for theobromin (Fig. 1), cinchonin (Fig. 4), quinin (with *Gammarus*) (Fig. 4), quinin (with paramœcia) (Fig. 6), veratrin (Fig. 7), and colchicin (Fig. 7) each show *two* distinct maxima of toxicity; while the curve for strychnin (Fig. 7) shows traces of a third maximum, and the curves for pilocarpin (Fig. 3) and for cocain (Fig. 6) each show *three* distinct maxima of toxicity. These phenomena would appear to be correlated in some way with the acid and basic properties of the alkaloids in question. Quinolin and caffein are both basic substances possessing no acid properties; theobromin is a monacid base, but the hydrogen which has been substituted for one of the methyl groups of caffein can be replaced by a metal, so that it also acts as a monobasic acid; leucin, as a monobasic amino-acid, acts both as a monacid base and as a monobasic acid; cinchonin is at the same time a diacid base and a monobasic acid; quinin is also both a diacid base and a monobasic acid; veratrin is a monacid base, but also forms salts with caustic alkalies; colchicin is a base, but is readily split up into a monobasic acid, colchicein, and methyl alcohol; strychnin is a monacid base, but there is reason to suspect the presence of a hydroxyl group; pilocarpin is a monacid base, but it dissolves in alkalies with the formation of salts of pilocarpinic acid—the formulæ of the copper and silver salts¹ appear to indicate that pilocarpinic acid is monobasic; cocain is a monacid base which is readily split up into the monobasic acid, benzoyl-ecgonin, and methyl alcohol, while benzoyl-ecgonin is decomposed on hydrolysis into ecgonin—which is at the same time a monacid base, a monobasic acid, and a monatomic alcohol, and is the saponification-product of cocain—and into benzoic acid.

Thus the only two alkaloids investigated which possess no acid properties, namely, caffein and quinolin, are also with the exception of leucin the only two which show only *one* maximum of toxicity in the different electrolytes. With the exceptions of pilocarpin and leucin and the possible exception of strychnin,

¹Vide Beilstein, *loc cit.*, iii, p. 924.

all the alkaloids which at the same time act as bases and as monobasic acids show *two* maxima of toxicity. While the only alkaloid investigated which can act at the same time as a base and, upon hydrolysis, as a monobasic acid and a monatomic alcohol (with the accompanying power of forming salts and alcoholates), namely, cocain, shows *three* maxima of toxicity.

I have suggested that the toxicity of an alkaloid depends upon the amount of the alkaloid which enters into combination with some constituent of the tissue, possibly ion-proteid. The greater the "acidity" of this constituent, that is, the greater its power of combining with a base, the greater would be the quantity of a basic alkaloid with which it would combine, and the greater would be the toxicity of the alkaloid.

The hypothesis as to the formation of ion-proteid compounds which I have outlined above leads us to conclude that the excess (A-B) of acid ion-proteid molecules is given by $0.5 - \frac{u}{u+v}$ or by $\frac{v}{u+v} - 0.5$. This quantity, therefore, indicates the power of an alkaloid which is purely basic to combine with the ion-proteids which are formed when an organism is acted upon by a given electrolyte. This is simply analogous to the case of an acid combining with a given amount of base; the concentration of the base, (in this case an alkaloid) is constant, while the concentration of the acid (in this case ion-proteid) varies.

If we construct a curve in which the abscissæ are proportional to the amounts of acid which we add to a given concentration of an alkali, while the ordinates represent the amount of the acid which combines with the base, the resulting curve has only one maximum (see dotted curves in Plate II, Fig. 5).

When the alkaloid is both an acid and a base, the case is analogous to adding both acid and base to a mixture of acid and base, and may be represented by combining *two* curves of neutralization—the one of a given amount of acid by different amounts of base, and the other of a given amount of base by varying amounts of acid. Fig. 5 represents such a compound curve. Starting from the extreme left where the dotted curve, I, cuts the base-line, the abscissæ are proportional to the molecular concentrations of alcohol mixed with a molecular solution

of acetic acid; the ordinates of the dotted curve, I, represent the amount of ethyl acetate formed.¹

The neutralization-curve is of the same general form in other cases, only the equilibrium-constant of the reaction is different. Starting from the extreme right where the dotted curve, II, cuts the axis, the abscissæ measured to the left represent molecular concentrations of acetic acid mixed with a molecular solution of alcohol, while the ordinates of the dotted curve, II, represent as before the amount of ethyl acetate formed. The ordinates are all made half the height of those in Curve I, in order to allow in some measure for the possibility, in a case which would be truly analogous to the one under consideration, of a different equilibrium-constant for this reaction.

Transferring the origin to the centre as represented in the diagram, and considering x as positive when measured to the right, it is obvious that $x = \frac{1}{2}(A - B)$ if A be the number of acid molecules present and B the number of basic molecules.

Returning to the case of an alkaloid which is at the same time a monacid base and a monobasic acid acting on a mixture of acid and basic ion-proteids, if both the alkaloid combined with acid ion-proteid and that combined with basic ion-proteid exert their toxic action, the toxicity should be represented by the sum of the amounts combined—that is, returning to our analogous case, to the sum of the ordinates of two curves such as I and II. Constructing therefore, a third curve by adding the ordinates of I and II, we obtain the compound curve, III, the similarity of which to the curves of toxicity of cinchonin and of quinin for *Gammarus*, (Fig. 4) with different values of $\frac{v}{u+v} - 0.5$ or $A - B$, cannot fail to be observed. The similarity extends to details; the fall at the extreme ends of the curves can be attributed, as in the analogous curve, to the somewhat sudden decrease in the amount of ester formed as the concentration of one of the components of the reaction approaches zero;

¹ The figures up to 1 molecular alcohol are taken after Berthelot, *Ann. d. Chim. et d. Phys.*, 3me Série, lxxviii, p. 225, 1863, and van't Hoff, *Ber. d. deutsch. chem. Gesellsch.*, x, p. 669, 1870, from Cohen's *Physical Chemistry* translated by Fischer, p. 73, 1903. The figures for higher concentrations of alcohol or acid are taken from Berthelot, *loc. cit.*, pp. 290 and 291.

the sudden changes in slope of the curves of toxicity also suggest those of the neutralization-curves whether simple or compound.

It will be seen also that three maxima could not be obtained in the compound curve unless three simple curves were employed—that is, unless we had a substance which was at the same time a monacid base and a dibasic acid, or a diacid base and a monobasic acid, acting upon mixtures of acid and base with different equilibrium-constants for each of the two reactions which are similar in nature,—for example, the neutralization of each of the two acid affinities of the dibasic acid. In harmony with this we have the fact that cocain shows three maxima of toxicity. Pilocarpin, however, which apparently can only act as a monobasic acid and a monacid base, also shows three maxima, and strychnin also shows traces of a third maximum.

The experiments, therefore, appear in general to afford results in harmony with those indicated by the hypothesis which I have outlined as to the formation of ion-proteids and its relation to the toxicity of alkaloids. If this hypothesis be correct, then the number of basic affinities, at least in the alkaloids investigated, does not appear to influence the curve of toxicity in the same way as the number of acid affinities—for cinchonin and quinin show only two maxima while they are diacid bases. Possibly the equilibrium-constants for the neutralization of each of the basic affinities in these cases are equal—as a third maximum would not then be obtained.

(6) *Some Possible Objections.*

It might possibly be objected that inasmuch as the acidity or alkalinity of the solutions of the various electrolytes used, due to hydrolytic dissociation, runs closely parallel with the differences in the transport-numbers, the effects observed might be due to specific actions of the hydrogen or hydroxyl ions present in the solution upon the toxic action of the alkaloids. This objection is answered by the results of the experiments on the influence of various mixtures of the mono- and disodic phosphates upon the toxicity of caffein and of quinin for *Gammarus* (Tables III, IV, XXVII, and XXVIII). Salm¹ has de-

¹ *Zeitschr. f. Elektrochem.*, x, p. 341, 1904.

terminated the concentration of the hydrogen ions in each of these mixtures; his figures are as follows:

Solution	I (50 c.c. Na_2HPO_4 , 0 c.c. NaH_2PO_4);	$\text{CH} = 1.3 \times 10^{-9}$
"	II (40 c.c. Na_2HPO_4 , 10 c.c. NaH_2PO_4);	$\text{CH} = 65 \times 10^{-9}$
"	III (30 c.c. Na_2HPO_4 , 20 c.c. NaH_2PO_4);	$\text{CH} = 190 \times 10^{-9}$
"	IV (20 c.c. Na_2HPO_4 , 30 c.c. NaH_2PO_4);	$\text{CH} = 490 \times 10^{-9}$
"	V (10 c.c. Na_2HPO_4 , 40 c.c. NaH_2PO_4);	$\text{CH} = 1500 \times 10^{-9}$
"	VI (0 c.c. Na_2HPO_4 , 50 c.c. NaH_2PO_4);	$\text{CH} = 33000 \times 10^{-9}$

It will be seen, on comparing with Tables IV and XXVIII, that the toxicities of caffein and of quinin vary quite irregularly with respect to the concentration of the hydrogen ions, an attempt to plot the curve revealing this very clearly; moreover, a maximum of toxicity of quinin occurs in Solution VI, for example, which is much more acid than calcium chloride. Caffein shows three maxima of toxicity in Solutions I, III, and VI, which does not correspond with the variation of its toxicity in the other salts employed if the variation be attributed to hydrogen and hydroxyl ions alone.

Another hypothesis which might possibly be advanced to explain these phenomena is that the salts alter the permeability of the cell-walls in different degrees so that the alkaloids cannot enter the cells with equal facility in different solutions of electrolytes. If this were so, however, we should have to suppose that the electrolytes alter the permeability in a different manner for each alkaloid, for we would be confronted with such facts as that the salt which renders the cells most permeable to pilocarpin, namely, barium chloride, is at the same time one of those which renders them least permeable to quinin or to veratrin; it is difficult to conceive how this would be possible. Referring the explanation to a physical factor, such as permeability, neglects the chemical factor which enables protoplasm to take up large quantities of one alkaloid or to appear indifferent to another, under otherwise similar conditions, according to the *chemical* differences between the two alkaloids. A still more emphatic experimental denial of the permeability hypothesis as a complete explanation of differences of staining-power brought about by different electrolytes may be found in my second communication on this general subject.¹

¹ *Loc. cit.*

While I do not by any means deny that other variables may exist besides those considered—indeed, the complexity of some of the curves, as, for example, the curve for pilocarpin, would appear to indicate the existence of such variables—I think that the experimental evidence is such as to lend support to the hypotheses I have advanced. Alterations of permeability, if they occur, must, of course, play a part, although the influence which they exert must, in my opinion, be in the same sense for each alkaloid. Hydrolytic dissociation must, of course, exert some influence, though it may be comparatively slight, inasmuch as hydrolytic dissociation must alter the proportions of kations and anions entering the protoplasm.

(7.) *Chemical and Physiological Meaning of these Results.*

If the experimental results which I have described in this paper, together with those on the influence of electrolytes upon staining-power in methyl green and in iodine-eosin, are sufficient to enable us to form any conclusion, they must be considered, I think, to afford additional evidence of the existence of unstable ion-compounds in protoplasmic bodies. If this be granted, then we must conclude that the ion in this compound is readily substituted by other ions for each of the electrolytes, the action of which has been investigated, exerts an influence in a definite sense upon the toxicity of an alkaloid or upon the staining-power. We can recognize the existence of these different compounds by the differences in the physiological behavior of the organism, indicating that the different ion-compounds have different properties. That the ion in the compound exerts an influence upon the "pseudo-acid" or "pseudo-basic"¹ properties of the molecule is not unlikely, in view of the usual effect of introducing a strongly acid or basic radical into a comparatively inert molecule; and the results obtained are in the sense which would be indicated by such an hypothesis.

Physiologically, we might consider these results as affording an explanation of the extremely local action of many alkaloids. Apart altogether from any hypothesis as to the means by which electrolytes may influence the toxicity of alkaloids, it is evident that the alkaloid which exerts hardly any influence in a solution

¹ Gustav Mann, *Physiological Histology*, Oxford, p. 25, 1902.

of one electrolyte may exert a very powerful influence in a solution of another electrolyte, and the maxima of toxicity for different alkaloids are in very different electrolytes. The presence or absence of a given electrolyte or of given proportions of ions, therefore, may readily determine the degree of influence which an alkaloid exerts upon a given tissue.

(8). *Possible Applications to Special Physiological Investigation.*

These considerations open out to us the possibility of utilizing the local actions of various alkaloids to indicate the nature of the ion-proteids present in different tissues. We know, for example, that basophile granules are abundant in nerve cells,¹ and we may therefore assume that anion-proteids are abundant in these cells, and this may possibly afford an explanation of the intensely local action of nicotine upon nerve cells.² Potassium salts predominate in muscular tissue, and this may perhaps afford an explanation of the localized action of veratrin upon muscular tissue, for our results have shown that the toxicity of veratrin is high when kation-proteid is abundant. That we cannot reason direct from the results obtained with small organisms bathed for a long time in a large volume of electrolyte to those obtained when muscles, for example, are bathed for a comparatively short time in a solution, is shown by the fact that, whereas calcium chloride and barium chloride favor the action of veratrin upon muscle, the toxicity of veratrin for *Gammarus* is at a minimum in barium chloride (Tables XXXV and XXXVII). We have, in fact, to take into consideration the influence of the salts in the muscle itself.

We may safely assume that the same amounts of different alkaloids combined with the ion-proteids of a cell will have different toxicities. Calling the toxicity of some unit of mass of the alkaloid combined with ion-proteids in a cell the "specific toxicity" of the alkaloid, it is evident that an alkaloid of low "specific toxicity" would not begin to exert a marked influence until the amount combined was pretty high. Hence such an alkaloid would only affect localities where it could enter into combination in comparatively large quantities. On the other

¹ Vide Gustave Mann, *loc. cit.*, p. 289.

² Langley, *Journ. of Physiol.*, xi, p. 123, 1890.

hand, although the primary and most intense effect of an alkaloid with a high "specific toxicity" would be where it can combine in the greatest mass, yet it might be expected to exhibit distinct secondary effects. Strychnin may possibly be such an alkaloid. Again, an alkaloid of "low specific toxicity" might exert in dilute solution an exciting action upon a physiological process and in concentrated solution a depressing one. By combining with an ion-proteid, the ion of which is inhibiting the process, a dilute solution might accelerate it, while a concentrated solution of the same alkaloid would exert its own toxic action. By comparison of simpler and comparatively known cases—as when small organisms are acted upon by dilute solutions of the alkaloid so that the influence of the salts already present in the organism can be neglected—with the more complex cases which occur in the vertebrate system, it would appear possible, with the assistance of staining methods, to obtain definite information as to the nature of the ion-proteids present in a given tissue.

(9). *Possible Applications to Therapeutics.*

An exact knowledge of the influence of saline media upon the action of alkaloids cannot fail to be of importance in therapeutics, and if it be found that other drugs with distinctly acid or basic characters are similarly influenced by electrolytes, it may ultimately be possible to modify and control the actions of various drugs by the simultaneous administration in a suitable manner of appropriate saline solutions. The more effective control which we would thus obtain over the actions of these drugs would enable us to produce a greater variety of effect. Our power in this direction would, of course, be limited by our power of influencing the proportions of the different ions in a given tissue by altering the proportions in the blood, but these experiments have shown how marked an influence even slight differences in the relative proportions of the kation- and anion-proteids may have upon the action of an alkaloid.

The experiments on the influence of electrolytes upon the toxicity of quinin for infusoria are of especial interest because of the importance of a thorough knowledge of the conditions affecting the action of quinin upon the plasmodium in malaria. It would appear as if it might be possible to increase the toxicity

of quinin for the plasmodium by slightly altering the proportion of anions to kations in the plasmodia in the blood. The chief salt in the blood is sodium chloride, which is the salt in which quinin is most toxic for *Gammarus*, but the ratio of anions to kations which would diffuse into the cells from the blood is lowered by the presence of potassium salts. By the addition of magnesium chloride or of sodium sulphate the ratio could be raised. Whether the injection of solutions of these salts in the requisite amounts would have other and undesirable effects is a question for physicians to decide.

IV. CONCLUSIONS.

1. The influence of various salts upon the toxicity of fourteen alkaloids for *Paramœcium*, *Tubifex*, and *Gammarus*, and the influence of some salts upon the action of veratrin upon striated muscle have been investigated.

2. The various salts exert a distinct and definite action upon the toxicity of the alkaloids investigated.

3. The effects observed are not due entirely to alterations in permeability or to the influence of hydrions or hydroxidions set free by hydrolytic dissociation of the salts.

4. The results are such as to lend support to the hypothesis of the existence of an unstable ion-compound in protoplasm, the ion of which is readily replaced by other ions, the ion which is present in the greater mass forming the greater part of the compound, the acid or basic properties of which depend upon the acid or basic properties of the ion in the compound.

5. Certain instances were found in which the toxicity of a salt was diminished by the addition of the alkaloid, while the same alkaloid in the same concentration increased the toxicity of other salts. These cases may possibly be attributed to the alkaloid removing or neutralizing a toxic ion by combining with the ion-proteid which it forms.

6. Possible applications to special physiological investigation and to therapeutics have been indicated.

Finally, I wish to express my indebtedness to Dr. Loeb for the numerous facilities and the encouragement and advice which he has extended to me in carrying out these experiments; also my indebtedness to Dr. F. G. Cottrell for valuable suggestions.

Figure 1.

Toxicity

Caffeine
Theobromine

$$(0.5 - \frac{r}{u+v})$$

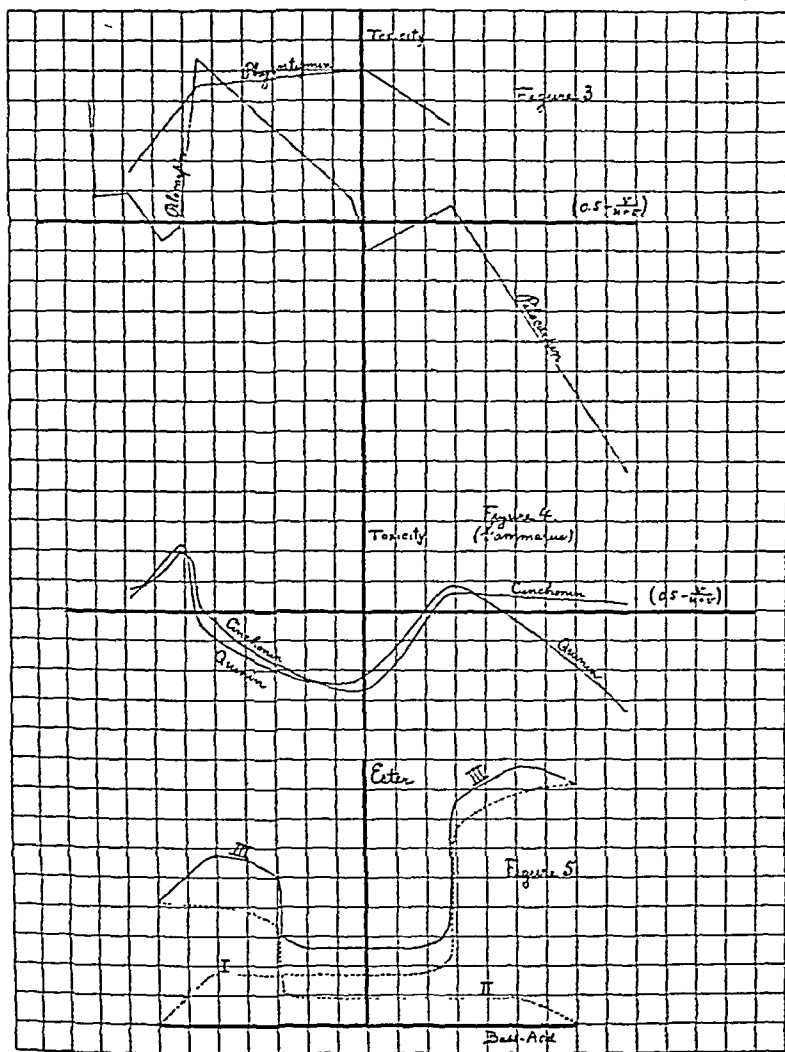
Figure 2.

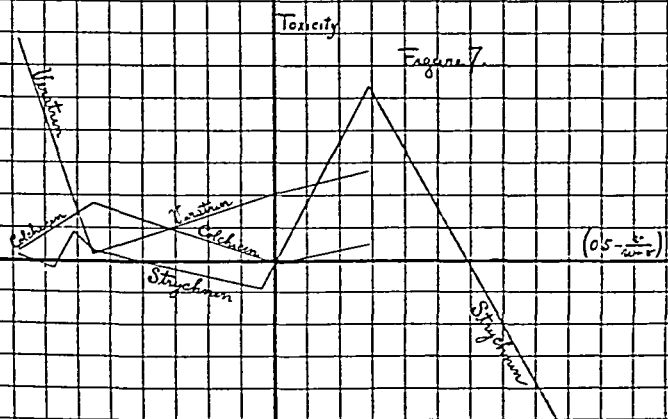
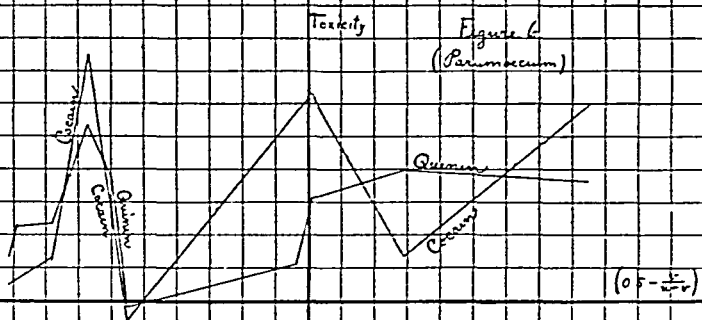
Toxicity

Quinine

Lucifer

$$(0.5 - \frac{r}{u+v})$$





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